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I. THE RATE OF PROTEIN CATABOLISM[1].

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The whole question of the course of the breakdown of the protein molecule within the tissues is one of the most obscure in physiology. We have now a fairly good idea of the form in which the protein is absorbed from the lumen of the intestine but its immediate fate is still unknown. Apparently however definite evidence is now collecting [Folin, 1912] in support of the view that no immediate synthesis, analogous to that of fat, takes place. Unquestionably, irrespective of the form in which the protein material is conveyed to the tissues, there is, soon after the ingestion of food, a fairly complete disintegration of the protein molecule as evidenced by the increase in the output of nitrogenous substances in the urine. Of course it might be maintained, as it has been, that this material arises not from the newly ingested material but from "effete" protoplasm broken down and discarded when a new supply of repair material is available. If this be so then the material excreted ought to bear some definite percentage relationship to the normal protein of the body, for example the ratio of sulphur to nitrogen in the urine should approximate to that of the average tissue as obtained say by the study of the sulphur and nitrogen ratio in complete starvation.

It was thought that, by a careful study of the ratios of S : N after feeding with specially chosen foodstuffs, light might be thrown on :—

     (1)   The rate of protein catabolism.

     (2)   The nature of the material catabolised.

PREVIOUS WORK.

Little work has been carried out in this field probably because of the fact that until recently the difficulty of carrying on a long series of sulphur analyses was considerable. Since the introduction of the very excellent and rapid method of S. R. Benedict this difficulty has largely disappeared.

[1] The majority of the results here published were given in the form of a communication at the Biochemical Society's Cambridge meeting, October 1911.

Siven [1901] found that the sulphur output ran closely parallel to the nitrogen output. He came to the conclusion that all the nitrogen excreted does not come from a purely protein source.

Sherman and Hawk [1901] also found that the nitrogen and sulphur outputs ran very closely parallel.

Von Wendt [1905] made a very thorough investigation into the output of both nitrogen and sulphur. He found that when protein was broken down in the body, the sulphur-rich digest products were the first to be catabolised, i.e. that the sulphur output preceded that of the nitrogen and that therefore the nitrogenous products which were retained in the tissues were comparatively poor in sulphur. Von Wendt maintained that it is only when the nitrogen and sulphur excretions are considered together that the true picture of the total protein exchange in the body can be obtained : individually considered they only tell whether certain decomposition products are excreted.

Ehrström [1906] pointed out that, although the nitrogen and sulphur excretions ran practically parallel, the sulphur output acted more rapidly with any change in the intake. He suggested that the sulphur-containing amino-acids were more readily oxidisable in the organism and that this might account for the accelerated rate of output of the sulphur in comparison with that of the nitrogen.

Falta [1906] also supported the view that the protein molecule was catabolised in a step-like fashion. He believed that certain nuclei were more labile than others and that this lability would account for the appearance of certain products before other constituent substances of the protein molecule. He further pointed out in a very clear way that even in the case of the nitrogen-containing moiety of the protein molecule the output of the excess nitrogen or waste nitrogen was not immediate but that it might—depending on the nature of the material ingested—be spread over several days. Thus after the ingestion of caseinogen an amount of nitrogen equivalent to that taken in was excreted in four days whereas after the administration of egg albumin the excretion was continued to the sixth day.

Hämäläinen and Helme [1907] as a result of their experiments with a superimposed diet as used by Falta came to the conclusion that the output of sulphur ran very closely parallel to the output of nitrogen. At the same time they held that their results bore out fully the contention of von Wendt that the sulphur-rich products arising from the catabolism of the protein molecule are more rapidly burnt and excreted than those which are sulphur-poor.

Since our work was completed, two papers by Wolf [1912] have appeared which practically cover the same ground as our own. Wolf found that in some instances the maximum of the output of sulphur preceded that of the nitrogen but that in others the sulphur appeared simultaneously with or was behind the nitrogen. He arrived at the general conclusion however that in the majority of instances the sulphur part of the protein molecule is the site of the first catabolic process.

METHODS.

In our experiments we also adopted the superimposition method of feeding introduced by Falta, i.e. we added on a particular day to a standard diet the special foodstuff the rate of catabolism of which we wished to establish. The standard diet was always started four or five days previous to the test in order to get the organism into a state of approximate equilibrium. (It may be remarked here that in the majority of instances, particularly those on a low protein intake, the lowest level of nitrogen was probably never reached.) On a set day there was then added to the ordinary diet, as a rule with the morning meal, the special foodstuff. Then on the following two, three or four days the original diet was continued without addition of any kind. As far as possible throughout the feeding period the intake of fluid was kept constant. The subject H. H. G. lived an ordinary quiet laboratory life, taking no undue exercise.

The diets which we employed were (1) a low nitrogen diet consisting of tapioca and cream or potatoes and butter; (2) a diet containing a medium amount of nitrogen made up of bread, butter and milk and finally (3) a diet rich in nitrogen consisting of eggs, cheese, milk and bread.

As we were only concerned with the rate at which the nitrogen and sulphur were excreted the output of total nitrogen and total sulphur were alone considered. As a general rule, as the diets were creatine- and creatinine-free, the opportunity was taken of studying the output of these substances under the different conditions of the experiments. The total nitrogen was estimated by the ordinary Kjeldahl method and the total sulphur by Benedict's method [1909], creatine and creatinine by Folin's method.

In order to get as perfect a picture as possible, the outputs of total nitrogen and of total sulphur were estimated every two hours during the day of the experiment. As a rule, for purposes of comparison the estimations on the preday, and sometimes on the day immediately following, were also carried out on the two-hourly plan. The output of the total nitrogen in the faeces was also determined.

GELATIN.

Three experiments were carried out with gelatin superimposed on various diets, (1) tapioca, (2) potato diet, (3) the egg diet.

The first experiment was carried out with the tapioca diet which had been given for two days previously. The amount of gelatin given was that present in 515 grams .of a " table jelly," which contained seven grams of nitrogen and ·503 grams of sulphur, with therefore an S : N ratio of 1:13·9. The whole of the gelatin was taken with the breakfast meal at 9 a.m. The following table gives the result of the experiment.

TABLE I.

Gelatin feeding (tapioca diet).

Date and hour	Total nitrogen in grms.	Total sulphur in grms.	N : S ratio	Creatinine in grms.	Remarks
Sept. 16, 1910	9·78	·624	15·7	2·02	
,, 17, ,, 9–11	·568	·0356	16·0	·162	Two-hourly collection.
11– 1	·554	·0384	14·4	·168	
1– 3	·901	·0462	19·5	·181	
3– 5	·697	·0366	19·0	·162	
5– 7	·618	·0385	16·0	·177	
7– 9	·680	·0549	12·4	·182	
9–11	·506	·0292	17·5	·164	
11– 9	2·050	·1366	15·0	·718	
	6·574	·4160	15·8*	1·92	
Sept. 18, 1910, 9–11	·490	·0354	13·8	·170	Fed day.
11– 1	·643	·0505	12·7	·178	515 grms. "table jelly'
1– 3	·902	·0735	12·3	·177	=7 grms. N taken at
3– 5	·697	·0607	11·5	·166	9 a.m.
5– 7	·645	·0658	9·8	·166	
7– 9	·918	·0594	15·5	·187	
9–11	·838	·0395	21·2	·160	
11– 9	2·732	·1389	19·7	·693	
	7·865	·5237	15·0*	1·90	
Sept. 19, 1910, 9–11	·462	·0291	15·9	·162	
11– 1	·539	·0352	15·3	·180	
1– 3	·566	·0311	18·2	·170	
3– 5	·574	·0284	20·2	·168	
5– 7	·454	·0256	17·7	·162	
7– 9	·798	·0411	19·4	·176	
9–11	·532	·0286	18·0	·168	
11– 9	2·197	·1254	17·5	·706	
	6·122	·3445	17·7*	1·90	
Sept. 20, 1910	5·7	·3189	18·1	1·90	

* Ratio of totals.

When the two-hourly outputs of the preday and the fed day are compared it will be noted that in the case of the total nitrogen the alteration from the preday takes place late in the afternoon whereas with the sulphur the change occurs a few hours after the ingestion of the gelatin. From an examination of the S : N ratios the increased rate of the sulphur output over the nitrogen is clearly seen. Even at the end of the first two hours the ratio of sulphur to nitrogen has increased to 13·8 as compared with the ratio of the total outputs of the previous day of 15·8. As will be noted the output of sulphur accelerated over that of the nitrogen until the ratio stood at 9·8. Later on however the rate of the output of nitrogen suddenly increased with the result that the apparent lag was almost done away with, the ratio of S : N falling to 21·2. This gives an average for the total outputs of the sulphur and the nitrogen for the day of 15·0, a result but little different from the preday. The postday shows a fall to 17·7. As regards the total output of the super-imposed nitrogen and sulphur there is evidence of a marked retention of both substances. Of the ingested nitrogen only 18·6 % is excreted and of the sulphur 26·2 %. On neither of the two postdays is there any evidence of the excretion of the retained nitrogen, 5·7 grams.

Unfortunately no estimation of the nitrogen excreted by way of the faeces was carried out as previous experiments with other forms of superimposed foodstuffs had shown that no marked excretion took place by that channel. Still the retention here might have been only apparent, i.e. the bulk of the nitrogen might have been excreted by way of the faeces. Of course at the same time it was possible that a true retention of nitrogen had taken place especially as the subject was on a tapioca diet where a special demand for protein might be presumed.

In order to test this possibility the gelatin in the following experiment was superimposed on a diet rich in protein. The diet consisted of 10 eggs, 4 ozs. of cheese, 2 pints of milk, 8 ozs. of bread and 3 ozs. of butter. The gelatin employed was the same as in the previous experiment and the same amount was given. The only difference was that the subject had so much difficulty in consuming it with his otherwise abundant morning meal that about one third had to be left till the midday meal. Again, in order to get nitrogen equilibrium on the diet previous to the superimposition there were six predays of feeding (the urine on the sixth day being collected two-hourly). As will be seen from the following table the effect of the superimposition was not that of the previous experiment. Here there is neither a marked rise in the output of either the nitrogen or the sulphur nor is there an increase in the S:N ratio; indeed there is a slight decrease.

TABLE II.

Gelatin feeding (egg and cheese diet).

Date and hour		Total nitrogen in grms.	Total sulphur in grms.	N : S ratio	Creatinine in grms.	Remarks
Oct. 9, 1911		16·0	1·231	13·0	—	Weight 70·7 kilos.
,, 10, ,,		17·0	1·270	13·4	—	,, 70·9 ,,
,, 11, ,,		16·7	1·292	13·0	1·97	,, 71·0 ,,
,, 12, ,,	9–11	1·46	·1091	13·4	·165	,, 71·6 ,,
	11– 1	1·40	·0996	14·0	·178	Two-hourly collection.
	1– 3	1·54	·1036	14·8	·176	
	3– 5	1·48	·1042	14·2	·178	
	5– 7	1·46	·1041	14·1	·176	
	7– 9	1·76	·1295	13·6	·178	
	9–11	1·58	·1315	12·0	·165	
	11– 9	6·93	·5720	12·1	·755	
		17·61	1·349	13·0*	1·97	
Oct. 13, 1911,	9–11	1·55	·1179	13·2	·170	Weight 71·2 kilos.
	11– 1	1·66	·1266	13·1	·174	515 grms. "table jelly"
	1– 3	1·91	·1259	15·2	·168	with 7 grms. N taken ⅔
	3– 5	1·90	·1213	15·7	·176	at 9 a.m., ⅓ at 1 p.m.
	5– 7	1·89	·1135	16·7	·178	
	7– 9	1·92	·1122	17·1	·183	
	9–11	1·63	·1136	16·9	·179	
	11– 9	7·82	·5793	13·5	·736	
		20·28	1·410	14·4*	1·96	
Oct. 14, 1911,	9–11	1·61	·120	13·3	·179	Weight 71·6 kilos.
	11– 1	1·69	·113	14·9	·182	
	1– 3	1·62	·110	14·7	·179	
	3– 5	1·55	·112	13·0	·184	
	5– 7	1·52	·112	12·7	·176	
	7– 9	1·88	·143	13·1	·180	
	9–11	1·20	·133	10·9	·176	
	11– 9	7·60	·654	11·6	·775	
		18·67	1·497	12·4*	2·03	
Oct. 15, 1911		19·0	1·448	13·1	2·04	
,, 16, ,,		18·6	1·389	13·4	1·99	
,, 17, ,,		18·8	1·392	13·5	1·97	Weight 71·6 kilos.

* Ratio of totals.

If the normal output of nitrogen for the predays be taken as **17·1 gram**, the mean of the three predays preceding the superimposition, then practically the whole of the ingested extra nitrogen was excreted at the end of the second day after the feeding. 45·4 % of the extra nitrogen was excreted on the day of feeding, 22·4 % on the following day and 27·1 % on the second day after. For some inexplicable reason the output of nitrogen does not return to its old level.

In this experiment the output of nitrogen in the faeces was followed. The average output for four predays (collected and estimated each day) was 3·13 grams. The output on the day of feeding was 3·15 grams, on the first postday 3·72 grams, and the average daily output for the three following days was 2·59 grams. Evidently then the retention of the nitrogen in the first experiment was due to the actual retention of the nitrogen within the tissues and not to an excretion by way of the intestine.

As regards the output of the sulphur as already noted there is a slight fall in the ratio on the day of feeding to be followed however on the first postday by a slight rise; on the other postdays the ratio practically returns to the normal ratio found previous to the day of feeding.

If the normal output of sulphur be taken as the average of the four predays, viz. 1·31 gram, then on the day of feeding 21·8 %, of the ingested sulphur has been excreted, 35·8 %, on the first post-day, and 29·8 %, on the second. Thus the whole of the ingested sulphur, just as in the case of the nitrogen, is excreted within three days but also, just as with the nitrogen, instead of returning to its original level the output of sulphur continues slightly above normal. It is extremely difficult to offer any adequate explanation for this continued increase in the outputs of nitrogen and sulphur unless it be that the ingestion of the gelatin has stimulated protein catabolism.

Later a third experiment was tried in which there was a return to a lower nitrogen intake. The diet on this occasion consisted of boiled rice 10 ozs. (weighed dry), potatoes 1 lb. (weighed after peeling), butter 6 ozs., milk ½ pint, containing about 5·5 grams nitrogen. The day of superimposition was preceded by six days on the diet alone, the food being taken in four meals. On the seventh day of the diet 670 grams of a "table jelly" were superimposed. The subject found it absolutely impossible to consume this amount of gelatin at the first meal, and accordingly half the amount was taken at breakfast, one fourth at midday, and the other fourth with the afternoon meal. No attempt was made in this experiment to collect the urine two-hourly. The faeces were again examined for their output of nitrogen and the following figures show quite conclusively that no loss took place by this channel: average daily output on the predays 3·24 grams, day of feeding 3·22 grams, on days immediately following 3·12 grams and 3·24 grams.

As regards the output of nitrogen in the urine it is found that there is an immediate rise on the day of feeding and that there is also a distinct increase in the output during the three following days. The same statement holds

TABLE III.

Gelatin feeding (rice, potatoes, butter and milk diet).

Date	Total nitrogen in grms.	Total sulphur in grms.	N : S ratio	Creatinine in grms.	Weight, kilos.	Remarks
March 19, 1912	8·53	·658	12·9	—	—	
20	7·82	·678	11·6	—	72·2	
21	7·02	·556	12·4	1·94	72·3	
22	6·85	·582	12·3	1·92	—	
23	6·72	·547	11·7	1·92	72·0	
24	6·60	·564	11·7	1·92	71·8	
25	10·18	·883	11·4	1·91	—	On the 25th 670 grms. "table
26	9·07	·663	13·7	1·96	72·0	jelly" taken (= 9·12 grms. N)
27	7·60	·663	11·5	1·94	—	½ at 8.30 a.m., ¼ at 1.30 p.m.
28	7·14	·582	12·3	—	—	& ¼ at 5 p.m. Urine collected
29	6·60	·560	11·8	—	71·9	from 8.30 a.m. to 8.30 a.m.

good for the output of sulphur during these days. There is a curious irregularity in the S : N ratio which cannot be accounted for. The results were all checked by repeat analyses and found to be correct.

As regards the percentage amount of the ingested nitrogen and sulphur which was excreted during the day of feeding and the three following days the table below gives the result very clearly.

TABLE IV.

	N	S
	Percentage	excreted
Fed day	37·9	59·0
First day after	25·7	18·5
Second ,, ,,	9·6	18·5
Third ,, ,,	4·6	3·9
	77·8	99·9

Thus it will be noted that there is again quite a well-marked retention of nitrogen although it does not take place in the case of the sulphur. This retention is not so conspicuous as that which took place in the first experiment. The difference in result may be due to the fact that in the second experiment although the protein content is comparatively low still the diet is a more mixed one. Both experiments demonstrate that the sulphur, as other observers have previously found, is excreted more rapidly than the nitrogen.

EGG ALBUMIN.

Two experiments were carried out with boiled egg-white superimposed in the first case on the tapioca diet and in the second on the potato and butter diet.

In the first experiment the tapioca was given for two days previously, the urine on the second day being collected two-hourly. On the third day 8 ozs. of boiled egg-white containing 4·75 grams nitrogen and ·594 gram sulphur, thus with a S : N ratio of 1 : 8, were taken with the first meal. The collection of the urine was continued on the tapioca diet for still another day. The results obtained are given in the following table.

TABLE V.

Egg-albumin feeding (tapioca diet).

Date and hour	Total nitrogen in grms.	Total sulphur in grms.	N : S ratio	Remarks
Aug. 18, 1911	8·81	—	—	—
„ 19, „ 9–11	·68	·0374	18·0	Two-hourly collection.
11– 1	·64	·0394	16	
1– 3	·63	·0344	19	
3– 5	·83	·0436	19	
5– 7	·78	·0428	18	
7– 9	·72	·0507	14	
9–11	·38	·0266	14	
11– 9	2·17	·1317	16	
	6·83	·4066	17*	
Aug. 20, 1911, 9–11	·344	·0266	13	225 grms. boiled egg-white
11– 1	·406	·0340	12	(=4·75 grms. N) superim-
1– 3	·589	·0437	13·5	posed at 9 a.m.
3– 5	·813	·0710	11·4	
5– 7	·675	·0580	11·7	
7– 9	·874	·0827	10·6	
9–11	·788	·0691	11·4	
11– 9	2·070	·1870	11·1	
	6·56	·5721	11·4*	
Aug. 21, 1911, 9–11	·399	·0392	10·2	
11– 1	·382	·0416	9·2	
1– 3	·580	·0419	14·0	
3– 5	·473	·0409	11·5	
5– 7	·468	·0353	13·3	
7– 9	·474	·0366	13·0	
9–11	·672	·0425	15·8	
11– 9	2·090	·1356	15·4	
	5·54	·4136	13·4*	

* Ratio of totals.

It will be noted that the total output of nitrogen is actually less on the fed day although there is an increase in the output of sulphur. When the two-hourly outputs of the preday and the feeding day are compared it is seen that the diminution in the output of the nitrogen is due to some

apparent retention during the early hours of the day. Although the rise takes place on both days about the eighth hour, on the fed day instead of a diminution following there is a marked increase, particularly at the twelfth and fourteenth hours. During the night period however there is less nitrogen excreted than during the night of the preday.

As regards the sulphur output on the fed day there is also less sulphur excreted during the first four hours than during the same period on the preday but thereafter there is a well-marked rise in the output. This acceleration in the rate of the output of sulphur is particularly noticeable when the $S:N$ ratios are compared with those of the preday. The actual maximum on the preday is 14, whereas on the fed day it rises to 10·6.

There is no rise in the output of nitrogen on the day following although the output of sulphur still shows some increase.

In this experiment it is clear that under the conditions employed there has been a complete retention of the 4·75 grams of nitrogen given, but, of the ·594 gram sulphur, ·173 gram has reappeared, i.e. 29 % of the amount ingested.

A second experiment was carried out in which 11 ozs. of boiled egg-white containing 6·5 grams nitrogen and ·81 gram sulphur with a $S:N$ ratio of $1:8$ were superimposed on a potato and butter diet containing about 4 grams of nitrogen (3 lbs. potatoes and $\frac{1}{2}$ lb. of butter). In this experiment four days of the diet were carried out and then the egg-white was taken with the morning meal on the fifth day. The diet was continued and the urine collected for two days more.

The results will be found in Table VI (page 11).

Here it will be noted that there is not a complete retention of the ingested nitrogen although as in the previous experiment there is quite a well-marked rise in the output of the sulphur. When the two-hourly outputs of the fed day and the preday are compared it is found that the outputs of nitrogen bear a very close resemblance to one another. In each the maximum output takes place about the eleventh and twelfth hours. In the case of the sulphur the output rapidly rises and continues well above the preday throughout. This accelerated output of sulphur is also clearly demonstrated when the $S:N$ ratios are examined. The maximum ratio on the preday is 10 whereas on the fed day it rises to 7·6, the total ratio for this day compared with that of the preday being 9·8 to 12.

On the day following there is no continued rise in the output of nitrogen and the sulphur output also falls back practically to the preday level, although the $S:N$ ratio is still high, 10·7, indicating a continued acceleration

in the output of the sulphur. The second day after the feeding shows a further fall in the output of nitrogen and also in that of the sulphur. The S : N ratio is however still high, being 10.

TABLE VI.

Egg-albumin feeding (potatoes and butter diet).

Date and hour		Total nitrogen in grms.	Total sulphur in grms.	N : S ratio	Creatinine in grms.	Remarks
Sept. 3, 1911		9·8	—	—	—	
„ 4, „		8·3	·804	10·4	2·06	
„ 5, „		7·6	·685	11·1	1·97	
„ 6, „	9–11	·700	·0668	10·5	·174	
	11– 1	·767	·0688	11·1	·182	
	1– 3	·526	·0536	10·0	·170	
	3– 5	·743	·0597	12·4	·180	
	5– 7	·637	·0626	10·2	·180	
	7– 9	·980	·0653	15·0	·182	
	9–11	·764	·0584	13·1	·186	
	11– 9	3·100	·2482	12·5	·850	
		8·217	·6834	12·0*	2·10	
Sept. 7, 1911,	9–11	·768	·0604	12·6	·177	312 grms. boiled egg-
	11– 1	·788	·0653	12·1	·171	white (=6·5 grms. N)
	1– 3	·663	·0664	10·0	·170	superimposed at 9 a.m.
	3– 5	·786	·0974	8·1	·181	
	5– 7	·786	·1027	7·6	·173	
	7– 9	1·037	·0933	11·0	·173	
	9–11	·781	·0871	9·0	·169	
	11– 9	3·394	·3436	9·9	·737	
		9·011	·9162	9·8*	2·00	
Sept. 8, 1911,	9–11	·722	·0612	11·8	·168	
	11– 1	·755	·0728	10·4	·178	
	1– 3	·636	·0584	9·2	·176	
	3– 5	·522	·0538	9·7	·174	
	5– 7	·593	·0608	9·7	·182	
	7– 9	·805	·0755	10·6	·188	
	9–11	·590	·0584	10·1	·169	
	11– 9	2·721	·2442	11·1	·734	
		7·344	·6851	10·7*	1·97	
Sept. 9, 1911		6·70	·676	10·0	1·97	

* Ratios of totals.

Thus of the 6·5 grams of nitrogen taken in (the mean of the three pre-days' output of nitrogen being 8·01 grams and of the sulphur for two days ·684 gram) there has only been 1 gram or 15·3% excreted and that on the day of feeding. Of the sulphur ·232 gram or 28·6% was excreted on the day of feeding and a mere trace on the first day after.

These results do not agree very well with those obtained by Wolf. He found that the sulphur output lagged behind that of the nitrogen. He had previously observed the same curious inversion of the output rates in his feeding experiments with uncoagulated egg albumin. This delay in the output of the sulphur did not take place however when predigested egg albumin was fed; in this case the sulphur output preceded that of the nitrogen.

We had also carried out a couple of experiments with uncoagulated egg albumin but we had used instead of the ordinary raw material from eggs a finely powdered dry product (Egg albumin extra fine powder, Merck). In both experiments the albumin was superimposed on a tapioca diet. As these experiments were amongst our earliest they were not so thoroughly carried out as those previously described. In the first, in which 6·7 grams of nitrogen were given, there was a retention of at least 5·3 grams nitrogen. (The sulphur output was not measured.) In the second experiment, in which the same amount of nitrogen was given, there was a retention of about 6·0 grams. In neither case was the urine collected on any day following the feeding. As regards the output of sulphur in the second experiment the total amount excreted rose above that of the preday, the S : N ratios altering from 14·3 on the preday to 11·5 on the day of feeding. In this experiment, although it was not very definite, there was some support for Wolf's contention that when uncoagulated egg albumin is fed the sulphur is at first not so rapidly excreted as the nitrogen, but that later, when the excretion does start, it proceeds at a faster rate than that of the nitrogen. Wolf believed that the sulphur complex in the uncoagulated egg albumin has some power of withstanding the onset of the ferments.

PLASMON.

Only one experiment was carried out with this substance in which it was superimposed on a bread, butter and milk diet. After twelve predays of feeding, 5·9 grams of nitrogen in the form of plasmon were superimposed daily for eight days with the result that there was a consistent retention of nitrogen throughout. The mean daily output of nitrogen before the plasmon was added was 15·6 grams in the urine and faeces and despite the daily extra intake of 5·9 grams during the plasmon period the mean daily total output was only about 17·4 grams, giving a retention of nitrogen during the whole plasmon period of about 33 grams nitrogen. The four days, which made up the after-period, did not show very conspicuously the slow fall, which was to

be expected from previous work, in the output of nitrogen to the original level. It may be noted that although the subject was in very good condition throughout this experiment there was a steady although slight gain in body weight. The sulphur output showed nothing abnormal. Wolf in his plasmon experiment also found that there was no definite evidence in this case that the sulphur output preceded the output of nitrogen.

TABLE VII.

Veal feeding (bread and butter diet).

Date and hour	Total nitrogen in grms.	Total sulphur in grms.	N : S ratio	Creatinine in grms.	Weight, kilos.	Remarks
Feb. 19, 1912	9·76	—	—	—	72·3	
,, 20, ,,	8·62	·625	13·8	1·89	72·4	
,, 21, ,,	7·90	·662	11·9	1·96	72·5	
,, 22, ,,	7·01	·616	11·4	1·96	72·5	
,, 23, ,,	7·73	·664	11·6	1·93	72·6	
,, 24, ,, 8–10	·575	·0436	13·2	·162	72·8	
10–12	·731	·0586	12·5	·178	—	
12– 2	·582	·0477	13·0	·172	—	
2– 4	·605	·0452	13·4	·176	—	
4– 6	·583	·0460	12·7	·178	—	
6– 8	·732	·0662	11·1	·176	—	
8–10	·680	·0663	10·3	·180	—	
10– 8	2·910	·2583	11·3	·779	—	
	7·40	·629	11·7*	1·99		
Feb. 25, 1912, 8–10	·579	·0482	12·0	·175	73·0	226 grms. stewed veal
10–12	·847	·0592	14·3	·206	—	(=10·6 grms. N) su-
12– 2	·991	·0574	17·3	·206†	—	perimposed at 8.30
2– 4	·964	·0614	15·7	·196	—	a.m.
4– 6	1·131	·0773	14·6	·200	—	† Trace of creatine
6– 8	1·360	·0945	14·4	·191	—	present.
8–10	·983	·0801	12·3	·179	—	
10– 8	3·577	·3380	10·9	·750	—	
	10·43	·8081	12·9*	2·10		
Feb. 26, 1912, 8–10	·882	·0710	12·4	·179	73·0	
10–12	·929	·0702	13·2	·184	—	
12– 2	·838	·0611	13·7	·180	—	
2– 4	·778	·0616	12·6	·181	—	
4– 6	·692	·0583	11·9	·177	—	
6– 8	·809	·0710	11·4	·180	—	
8–10	·723	·0625	11·4	·182	—	
10– 8	2·932	·2658	11·0	·792	—	
	8·58	·7215	11·9*	2·05		
Feb. 27, 1912	8·16	·668	12·2	1·96	72·7	
,, 28, ,,	7·87	·630	12·5	1·92	72·6	
,, 29, ,,	7·34	·596	12·3	1·90	72·6	

* Ratios of totals.

VEAL.

In this experiment 226 grams of veal containing 10·6 grams of nitrogen and ·664 gram of sulphur with a S : N ratio of 1 : 16 were superimposed on a bread and butter diet which was taken for six days previous to the day of feeding. The sixth preday urine was collected two-hourly. Following the day of feeding there were four postdays. Table VII (p. 13) gives the general result.

Here it will be noted that there is a well-marked rise in the excretion of nitrogen as the result of the feeding, an excretion which is spread over the day of feeding and the three following days. In the case of the sulphur there is also a rise in the output but the normal is regained at the end of the third day after feeding. When the percentage amounts of the intake of both nitrogen and sulphur are compared it is found that here there is no, or perhaps only a very slight, acceleration in the output of the sulphur as is seen from the following table.

TABLE VIII.

	N	S
	Percentage output	
Day of feeding	28·6	27·0
First day after	11·2	14·0
Second ,,	7·2	5·0
Third ,,	4·4	—

Thus of the nitrogen and sulphur taken in, 56·1 % (including 4·7 % in the faeces) of the nitrogen is excreted in four days, and 46 % of the sulphur in three days.

This result fully substantiates the result obtained by Wolf who found that about 45 % of the nitrogen taken in the form of veal was re-excreted within two days. He also found that the nitrogen and sulphur output ran nearly parallel, although the output curve of the sulphur was a little steeper than that of the nitrogen, indicating that the former output was slightly more rapid. It does not however agree with the results obtained by Hämäläinen and Helme, who did not find any marked retention of the nitrogen and who found a much more rapid excretion of the sulphur than of the nitrogen.

UREA.

Finally a test with urea was carried out on a comparatively low protein diet to see if, when a nitrogenous material was used which we presumed could not take the place of protein, retention of nitrogen took place. After

giving a potato and butter diet for four days twelve grams of pure urea containing 5·6 grams of nitrogen were superimposed on the breakfast meal. We found that over 95% was excreted during the day of feeding and on the subsequent day. There was thus practically no evidence of retention. Wolf obtained a similar result: he found an excretion of nearly 97% in the first two days. This result does not favour the idea that urea can replace to a certain extent the protein of the diet. If this were possible it would have been expected that under the particularly favourable conditions of our experiment, urea given on a comparatively low protein diet, a certain amount of retention of nitrogen would have occurred. The ingestion of the urea had no influence on the output of sulphur.

CREATININE OUTPUT.

The opportunity was taken in the course of the above experiments to follow the output of creatinine as it was thought that the effect of the addition of a nitrogenous substance free from creatine and creatinine to a diet poor in protein might throw some additional light on the course of the metabolism of creatine and creatinine. Our results show that the output of creatinine was scarcely affected by such additions, except of course in the case of the veal which contained creatine in small amount. This substantiates the statement of Folin that the output of the creatinine is maintained practically at a constant level.

DISCUSSION OF RESULTS.

From these experiments then, and from others carried out by Wolf, etc., it may be concluded that Falta is right in his contention that the breakdown of protein takes place in a more or less step-like fashion. The fact that in nearly every instance there is an attempt by the cells of the organism to retain a certain amount of the nitrogen and to get rid at the same time of the excess of sulphur points to the fact that the form in which the nitrogen is retained, and apparently it is retained, although perhaps to a very limited extent, is a special one. The evidence for the storage of protein in the body is very scanty and has previously been discussed by one of us [E. P. C., 1912] at some length. Briefly it may be said that the general opinion is that a certain amount of retention can take place although the form in which the material is retained is still a matter of dispute. Some of the most interesting work in this connection is that of Müller [1907] who fed

dogs on a definite low protein diet and then amputated a limb. Subsequently the animal was fed on a high protein diet and killed after a long period of feeding. An analysis of the tissues obtained from the amputated limb on the low protein diet compared with the analysis carried out after the high protein diet showed that a certain amount of retention had taken place. Recently Diesselhorst [1911] repeated this work of Müller under practically the same conditions but making more elaborate analyses. He also found that there was a certain gain in the amount of nitrogenous material after feeding on the protein-rich diet. Grund [1910] also investigated the alterations in the composition of the tissues in starvation and after feeding. He discusses this question of the retention of reserve protein in the cells and comes to the conclusion that there is a certain amount of evidence in its favour, although he at the same time maintains that if this retention does take place it cannot play a very important part in the total metabolism. He further holds that there is a general tendency both in periods of starvation and of feeding for the tissues to maintain the same relative composition.

In conclusion we believe that from the study of the $S : N$ ratios it is clearly shown that the increase in the output of nitrogen and sulphur which as a rule follows the ingestion of a protein meal is due to the catabolism of the protein actually ingested and not to the displacement of "effete" proto-plasm from the tissues. It will be noted for example after the superimposition of the egg albumin with a $S : N$ ratio of $1 : 8$ (see Table VI) that the ratio obtained approximates very closely to the ratio of the ingested protein. This is particularly noticeable in the variations in the ratio in the two-hourly collection of the preday and the fed day. One of us [E. P. C., 1907] has previously shown that the $S : N$ ratio in starvation when all the nitrogen and sulphur which is excreted in the urine must come from an endogenous source is about $1 : 15$. If then the extra nitrogen and sulphur excreted after the ingestion of the egg albumin had come from the displacement of "effete" tissue protein it would have been expected that the $S : N$ ratio would be nearer $1 : 15$ than the $1 : 9\cdot8$ found.

Conclusions.

1. The sulphur-containing moiety of the protein after ingestion is, as a rule, more rapidly catabolised and the sulphur more rapidly excreted than the nitrogen.

2. When protein is superimposed on a low protein diet a retention of part of the nitrogen superimposed takes place.

3.   The retained material is apparently stored in the tissues as a pabulum of uniform composition.

4.   The rise in the output of nitrogen and sulphur after a protein meal is due to the catabolism of the actual material ingested.

5.   The superimposition of protein has, in these experiments, no effect on the output of creatinine.

REFERENCES.

Benedict (1909), *J. Biol. Chem.* **6**, 363.
Cathcart (1907), *Biochem. Zeitsch.* **6**, 109.
Cathcart (1912), *Physiol. of Prot. Metabolism*, 58, 80.
Diesselhorst (1911), *Arch. ges. Physiol.* **140**, 256.
Ehrström (1906), *Skan. Arch. Physiol.* **18**, 281.
Falta (1906), *Deut. Arch. klin. Med.* **86**, 517.
Folin and Denis (1912), *J. Biol. Chem.* **11**, 87.
Grund (1910), *Zeitsch. Biol.* **54**, 173.
Hämäläinen v. Helme (1907), *Skan. Arch. Physiol.* **19**, 182.
Müller (1907), *Arch. ges. Physiol.* **116**, 207.
Sherman and Hawk (1901), *Amer. J. Physiol.* **4**, 25.
Siven (1901), *Skan. Arch. Physiol.* **10**, 91.
von Wendt (1905), *Skan. Arch. Physiol.* **17**, 211.
Wolf (1912), *Biochem. Zeitsch.* **40**, 193.

II. THE FATE OF INDOLETHYLAMINE IN THE ORGANISM.

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It has been shown by several observers that the naturally occurring amino-acids are readily attacked by bacteria, and that among other products the corresponding amines are formed [Winterstein and Kuntz, 1909; Barger and Walpole, 1909; Ackermann, 1910; see Barger, 1911, for other references]. It appears probable that they are produced in the intestine in the course of the normal life of the organism and also in certain pathological conditions. Their fate after absorption is therefore a matter of considerable importance. The amines of this type have in most cases been submitted to physiological investigation [Dale and Dixon, 1909; Ackermann and Kutscher, 1910; Dale and Laidlaw, 1910], and in several cases they have been shown to be active substances. The method by which the body deals with such bases has not been investigated so thoroughly.

Some time ago we showed [Ewins and Laidlaw, 1910] that p-hydroxy-phenylethylamine was largely converted into p-hydroxyphenylacetic acid and excreted as such; that the liver and the plain muscle of the body, with the exception of the plain muscle of the lung vessels, could effect the change: and that successive methylation of the amino-group rendered the base increasingly resistant to destruction in the body. Another example of a change of precisely the same type is met with in the fate of benzylamine, which was shown by Mosso [1890] to be almost quantitatively converted into benzoic acid and excreted as hippuric acid. We undertook the investigation of the mode of destruction of indolethylamine (β-indole-pr-3-ethylamine) in order to see whether it was dealt with in a similar manner, and whether its administration would increase the kynurenic acid output in dogs.

The indolethylamine used was made according to the synthesis devised by one of us [Ewins, 1911], which readily gives a good yield of the base.

We had already shown [Ewins and Laidlaw, 1910] that it was possible to obtain this base by the action of putrefactive bacteria on tryptophane; but the method is tedious and troublesome, and the yield very poor.

We have carried out two sets of experiments in the course of our investigation: (1) feeding experiments on dogs, and (2) the perfusion of surviving livers of rabbits and cats[1].

PERFUSION EXPERIMENTS.

The method of perfusion was similar to that already described in our paper dealing with the fate of p-hydroxyphenylethylamine [Ewins and Laidlaw, 1910]. Small quantities of indolethylamine were perfused through surviving livers for 3—4 hours. The perfusion fluid gradually acquired the property of giving a fine red colour, when it was mixed with one-third of its volume of strong hydrochloric acid, with the addition of one drop of dilute ferric chloride, and then boiled. The red colour occasionally had a faint blue component if the hydrochloric acid was in excess or if the boiling was prolonged. A cherry red colour also developed if a trace of sodium nitrite was substituted for the ferric chloride in the above tests. Amyl alcohol rapidly extracted the pigment from the solution, when a well-defined absorption band in the green was seen with a spectroscope. The red colour is brighter and purer and the absorption band more intense and better defined when sodium nitrite is used. When the chromogen of the pigment appeared to be fairly abundant in the perfusion fluid (usually about 3–4 hours) the perfusion fluid was collected, and the liver vessels washed through with salt solution. The combined washings and perfusion fluid were rendered faintly acid with acetic acid and boiled. The coagulated proteins were filtered off and the filtrate evaporated to small bulk. The concentrated perfusion fluid was made acid to Congo-red with strong hydrochloric acid and shaken out with ether, which readily and completely extracted the chromogen. The ethereal extracts were combined, washed twice with water and taken to dryness. If the residue were taken up in water and allowed to stand, a small quantity of a crystalline acid was regularly obtained, which when recrystallised from benzene melted at 163–164°. These crystals gave the colour reactions mentioned above with great intensity and readily gave a deep orange-red coloured picrate melting at 174°. These properties taken together are characteristic of indoleacetic acid.

[1] The animal experiments were performed by P. P. Laidlaw only.

2—2

Experiment.   0·25 grm. indolethylamine hydrochloride was perfused through a rabbit's liver for 2·5 hours.   Perfusion fluid and washings were worked up as described above when 50 mgm. of indoleacetic acid were obtained.   Yield 44 % of theoretical.

The identification was completed by comparison of colour reactions, melting point, and mixed melting point with synthetic β-indole-*pr*-3-acetic acid.

There can be no doubt then that indolethylamine is readily converted by the liver into indoleacetic acid, just as *p*-hydroxyphenylethylamine is converted into *p*-hydroxyphenylacetic acid.

Feeding Experiments.

One half to one gram of the hydrochloride of indolethylamine is well borne by a 7–8 kilo dog.   Larger doses are inadmissible for our purpose since vomiting and sometimes purgation ensue.

The urine of the 36 hours following the administration did not give the colour reactions which we have always found in our perfusion experiments, but on adding to a small quantity of urine half its volume of strong hydrochloric acid and a trace of nitric acid and gently warming, a fine purple colour develops, which gradually (unless the mixture is cooled) becomes deep red and then orange and ultimately yellow.   The colour reminds one of a very intense indican reaction.   It is not, however, due to the development of indigo since at no stage can a blue component be extracted by chloroform.

We found that the chromogen of this colour reaction was an acid, which could be shaken out from the strongly acidified urine with ether or ethyl acetate, but the complete extraction of the chromogen by either of these solvents from acid urine was found to be almost impossible.   The acid, however, was found to be much more readily extracted if its solubility in urine was depressed by saturation with ammonium sulphate.   In a few experiments with rabbits' urine this preliminary saturation with ammonium sulphate was found to be of considerable assistance, since the procedure caused a large proportion of the hippuric acid to crystallise [cf. Roaf, 1908].

The ether or ethyl acetate extracts were carefully collected, and washed with ammonium sulphate, and then with a small quantity of water.   The acid was then extracted from the ether by sodium carbonate solution.   The alkaline extract was acidified, saturated with ammonium sulphate and shaken out with ether.   The ether extracts were washed with water and taken to dryness. A thick gum resulted from which nothing would crystallise.   The gum was

extracted with boiling water, in which most of it dissolved, excess of picric acid was added and the whole boiled with a small quantity of charcoal for five minutes. On filtration and prolonged standing the filtrate gradually deposited a deep orange-red coloured picrate. This picrate was recrystallised from water or water with a trace of acetone several times, when it separated in large, orange-red, rhomboidal plates melting at 145°. The crystals gave the colour reaction with great intensity. It appeared to us to be very probable that this acid would prove to be an indoleacetic-glycine complex, comparable with hippuric and phenaceturic acids, but for some time we were unable to obtain sufficient material to prove or disprove our assumption. The yield of picrate of the acid is not good and, as we have pointed out, the feeding experiments are limited to the administration of small quantities.

In one experiment for example, from 0·5 grm. of the hydrochloride of the base we only obtained 0·2 grm. of the picrate or a little over 20 % of the theoretical maximum yield. In some other experiments the yield was rather better but never more than 30 %.

From a series of three experiments we obtained sufficient of the picrate which, although not quite pure, could be analysed:

$$0·0986 \text{ grams picrate gave } 0·1668 \text{ } CO_2 \text{ and } 0·0322 \text{ } H_2O$$
$$C = 46·1 \qquad H = 3·6$$
$$C_{12}H_{12}O_3N_2 . C_6H_3O_7N_3 \text{ requires } C = 46·8, H = 3·2 \text{ per cent.}$$

The figures obtained although not accurate were sufficiently so to indicate that the acidic chromogen was an indoleacetic-acid-glycine condensation product, and that the substance in question has the probable constitution denoted by the formula
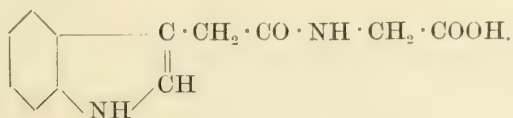
For this acid we suggest the name *indoleaceturic acid*, in conformity with the nomenclature of this class of bodies to which hippuric and phenaceturic acids belong.

It may also be mentioned that quite recently Ackermann [1912] has shown that among the products of metabolism of nicotinic acid fed to a dog is found a similar glycine derivative, to which he gives the name nicotinuric acid.

Our supposition with regard to the constitution of the urinary chromogen was further confirmed by hydrolysis of the picrate with sodium carbonate.

Experiment.   0·250 grm. of the picrate was dissolved in 80 c.c. of 5 % sodium carbonate solution and hydrolysed on the water bath for one hour. On cooling and acidifying, 0·19 grm. of indoleacetic acid picrate separated out, and from the mother liquor 0·017 grm. more was obtained crystalline. Total 0·207 grm. or 95 % of the theoretical amount if the original picrate had been the suggested indoleacetic-glycine complex.

Since the amount of acid obtainable in the experiments with indolethyl-amine was so very small, and we have shown by perfusion experiments that indoleacetic acid is probably an intermediate step in the production of indoleaceturic acid from indolethylamine, we carried out a series of feeding experiments with indoleacetic acid in the hope of obtaining larger quantities of the desired acid. Several grams of indoleacetic acid were synthesised by a method which will be published at a later date, and given by mouth to a dog, when a 60 % yield of picrate was obtained, identical in all respects with that obtained from the feeding experiments with the base. This fact enabled us to obtain the picrate in comparatively large quantities, since the indoleacetic acid caused no symptoms. In this way we obtained without trouble 3 grams of indoleaceturic acid picrate from 3 grams of indole-acetic acid. The preparation of the free acid from the picrate could not be carried out in the usual manner (removal of the picric acid from the acidified solution by means of ether) owing to the well-marked acidic properties of the indoleaceturic acid and its solubility in ether. This difficulty was overcome by the use of the base known as nitron (1.4-diphenyl-1.3.5-endanilino-dihydrotriazole) [Busch, 1905] which precipitates picric (as well as nitric) acid as an almost insoluble salt of the base. The experiment was carried out as follows.

1·5 grams of picrate were dissolved in 150 c.c. of hot water and 1 mole-cular proportion of nitron dissolved in hot dilute alcohol was added. The mixture was thoroughly cooled and the precipitated picrate sucked off at the pump. The nearly colourless filtrate was neutralised with sodium carbonate, two or three drops of acetic acid added and the solution evaporated to dryness. The residue was dissolved in a few (7–8) c.c. of water and made just acid to Congo-red with dilute hydrochloric acid. An oily product separated which soon crystallised in bunches of needle-like prisms. This was filtered off, dried in vacuo over sulphuric acid for a short time and weighed. In this way 0·6 gram of the crystalline acid was obtained. This was twice recrystallised from water and then melted at 94°. The acid so obtained is very slightly soluble in cold water (about 0·2 % at ordinary temperature), almost insoluble in ligroin and benzene, easily soluble in alcohol, ether and ethyl acetate. It

crystallises from water with one molecule of water of crystallisation which is only very slowly removed by sulphuric acid in vacuo at $37°$.

0·1022 gram acid (m.p. $94°$) gave 0·2184 CO_2 and 0·0514 H_2O

$$C = 58·2 \qquad H = 5·6$$

Calc. $C_{12}H_{12}O_3N_2 \cdot H_2O$ $\quad C = 57·6 \qquad H = 5·6$ per cent.

0·1026 gram acid (anhydrous) gave 0·2330 CO_2 and 0·0492 H_2O

$$C = 61·9 \qquad H = 5·3$$

Calc. $C_{12}H_{12}O_3N_2$ $\quad C = 62·0 \qquad H = 5·2$ per cent.

This result fully confirms our supposition that the acid in question is indole-aceturic acid.

It will be observed that we have never obtained anything like a quantitative yield of indoleaceturic acid by giving indolethylamine by mouth. In our best experiments about 30 % of the base is accounted for in this manner. It is true that this probably represents a much larger quantity of indoleacetic acid, which must be the intermediate product; because the administration of indoleacetic acid has never in our experience given a quantitative yield of indoleaceturic acid. However, making some allowance for this and a little for the imperfect methods used in the isolation of the acids from such a complex mixture as urine, we still have not accounted for the whole of the base.

It appeared to us to be possible that some of the indolethylamine or indoleacetic acid might be converted into kynurenic acid, although none of the specimens of urine that we had examined showed obvious excess of this metabolite. Some experiments were therefore undertaken in which kynurenic acid estimations were made before and after administration of indolethylamine and indoleacetic acid.

A dog was kept on a standard diet of milk and dog biscuit. On this particular diet the kynurenic acid excretion was minimal, and traces only could be detected in 24 hours. The kynurenic acid was estimated by Capaldi's method [1897].

Experiment.

Date	Volume of urine	Kynurenic acid	Remarks
Sept. 23—24	400 c.c.	Trace	—
Sept. 24—25	440 c.c.	Trace	—
Sept. 25—26	600 c.c.	·480 grm.	On morning of 25th 1·6 grm. tryptophane by mouth.
Sept. 30—Oct. 1	350 c.c.	Nil	
Oct. 1—2	600 c.c.	Trace	On Oct. 1st 1·6 grm. indoleacetic acid by mouth.
Oct. 2—4	850 c.c.	Traces	—
Oct. 4—5	470 c.c.	Traces	On Oct. 4th 1·0 grm. indolethylamine by mouth.

Neither indolethylamine nor indoleacetic acid increases the kynurenic acid output in dogs.

It seems clear that this path of metabolism of indole-derivatives does not aid us in accounting for the quantitative difference between the ingested substances and those excreted.

Samples of urine were examined in various ways for other possible end products but without success, and we suggest that the portion of the base unaccounted for is completely broken up in the body.

Occurrence of Indoleaceturic Acid.

We have some evidence of the occurrence of indoleaceturic acid in normal urine of herbivora. We have found that an acidic, ether-soluble substance, giving the purple colour reaction, occurs in small quantity in the urine of rabbits, but attempts at isolation of the acid or its picrate have always failed. The small amount of the chromogen cannot be separated from the other acid, ether-soluble substances present in all urines.

In examining urine for indican it occasionally happens that a fine purple colour is produced by hydrochloric acid and an oxidising agent but no blue component can be extracted by chloroform. Here, again, it is suggested that the chromogen is indoleaceturic acid, but considerable quantities of urine would be required to isolate the acid and such have not been at our disposal.

Herter [1908] described a case of a young girl who was suffering from some unusual intestinal infection and from whose faeces an unusual organism was isolated. The urine of the patient gave a marked urorosein reaction if the urine were stale. Indoleacetic acid was isolated from this urine and Herter identified it as the chromogen of urorosein originally described by Nencki and Sieber in 1882. The colour, solubilities and other characteristics of the pigment agree very well with those which Nencki and Sieber described as characteristic for urorosein, although other observers have described other substances as the chromogen of the same pigment. It is unfortunate that Herter gives no description of the method of isolation of indoleacetic acid from the urine of his patient. It is quite possible that it was originally present as indoleaceturic acid and that this was hydrolysed to indoleacetic acid in the process of isolation, for the complex acid is readily hydrolysed by weak alkalies such as sodium carbonate. The fact that stale urine gave a colour, while fresh did not, was attributed by Herter to the formation of nitrites. It is quite possible that the indoleaceturic acid was decomposed into indoleacetic acid and glycine, just as hippuric acid is readily split up by bacteria, and

that the nitrites, though certainly playing a part, did not furnish the whole explanation.

If it could be demonstrated that indoleaceturic acid was a normal constituent of urine it would by no means follow that its precursor in the body was indolethylamine, for bacteria often produce indoleacetic acid from tryptophane and proteins; in fact it is a more frequent product of bacterial action than indolethylamine.

SUMMARY.

1. Indolethylamine is converted, by the perfused liver, into indoleacetic acid.

2. Indoleacetic acid is excreted in combination with glycine forming indoleaceturic acid.

3. About 30 % of a given dose of indolethylamine is excreted as indoleaceturic acid in dogs.

4. Neither indolethylamine nor indoleacetic acid affects the kynurenic acid output in dogs.

REFERENCES.

Ackermann (1910), *Zeitsch. physiol. Chem.* **65**, 504.
Ackermann (1912), *Zeitsch. Biol.* **59**, 17.
Ackermann and Kutscher (1910), *Zeitsch. Biol.* **54**, 387.
Barger (1911), *Science Progress*, **6**, 221.
Barger and Walpole (1909), *J. Physiol.* **38**, 343.
Busch (1905), *Ber.* **38**, 859.
Capaldi (1897), *Zeitsch. physiol. Chem.* **23**, 92.
Dale and Dixon (1909), *J. Physiol.* **39**, 25.
Dale and Laidlaw (1910), *J. Physiol.* **41**, 318.
Ewins (1911), *J. Chem. Soc.* **99**, 270.
Ewins and Laidlaw (1910), *J. Physiol.* **41**, 78.
Ewins and Laidlaw (1910), *Proc. Chem. Soc.* **26**, No. 346.
Herter (1908), *J. Biol. Chem.* **4**, 239, 253.
Mosso (1890), *Arch. expt. Path. Pharm.* **26**, 267.
Roaf (1908), *Biochem. J.* **4**, 185.
Winterstein and Kuntz (1909), *Zeitsch. physiol. Chem.* **59**, 138.

III. THE HYDROLYSIS OF GLYCOGEN BY DIA-STATIC ENZYMES. COMPARISON OF PRE-PARATIONS OF GLYCOGEN FROM DIFFERENT SOURCES.

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For some time considerable uncertainty has prevailed as to whether samples of glycogen prepared from different sources are identical, the evidence on this point being in many respects contradictory. It is agreed that all have the same empirical formula $C_6H_{10}O_5$, the different results obtained by some observers being undoubtedly due to insufficient drying of their glycogen preparations. For Harden and Young [1902] found that the last traces of water can only be removed by heating to 100°C. *in vacuo* over phosphorus pentoxide, a procedure which had not been adopted by previous workers. Very divergent values have however been obtained for the specific rotation of different preparations of glycogen, the results covering a range of about 30°. Thus Cremer [1894] found as the $[\alpha]_D$ of yeast glycogen $+ 198.9°$, while Clautriau's [1895] result was 184.5°. Harden and Young [1902] also obtained for oyster and rabbit glycogens the value 191.2° while with yeast glycogen the mean of several determinations was 198.3°, a result in close agreement with that found by Cremer. It is uncertain however whether these differences are of any real significance, the strong opalescence of glycogen solutions making necessary the use of very dilute solutions and thereby introducing a large experimental error.

Other points of difference have however been noticed by various observers, though again the results are contradictory. Cremer for example states that yeast glycogen gives a darker colouration with iodine than does oyster glycogen, while other workers have obtained exactly opposite results. In a similar way different preparations of glycogen yield solutions with varying degrees of opalescence but these variations are by no means constant either in nature or extent. At present it is impossible to explain these results and

at the same time it is uncertain how far divergences of this nature imply any real differences between the respective glycogens.

A more valuable comparison was made by Harden and Young when they measured the rate of hydrolysis of different glycogen solutions by dilute acids. The course of hydrolysis was followed by estimations of the polarimetric and reducing powers of the solutions. By this method they were unable to detect any differences between rabbit, yeast and oyster glycogens and came to the conclusion that they were in all probability identical.

It seemed possible however that a study of the behaviour of various preparations of glycogen towards diastatic ferments might prove a more delicate means of detecting any difference in their constitution, for it has on many occasions been pointed out what marked influences even small alterations in the molecule may exert on the course of enzyme action. It is the results of such an investigation which are presented in this communication. Four preparations of glycogen have been employed, these being derived from (a) liver of dog, (b) liver of rabbit, (c) oyster and (d) yeast. The enzyme used was an extract of pig's pancreas, this being one of the most convenient sources for obtaining active preparations. Before making any direct comparison, the general conditions of action were studied, for although a great amount of work has been carried out on the behaviour of starch towards diastatic enzymes, the hydrolysis of glycogen has received very much less attention. These experiments will be first described.

Preparation of Glycogen.

A. Dog. A large dog was fed with considerable quantities of carbohydrate for forty-eight hours and then killed. The liver was rapidly removed, minced and heated on the water bath with 60 per cent. caustic potash. The glycogen was precipitated from this solution in the usual way [Pflüger, 1905] and purified by repeated reprecipitation from its aqueous solution, the first two or three precipitations being carried out with solutions rendered faintly acid with acetic acid. Glycogen was in this way obtained in considerable quantity, free from nitrogen and containing only a trace of ash. In the final stages it is frequently difficult to produce a satisfactory precipitation with alcohol alone, the addition of a small quantity of acetone in these cases, however, readily brings down all the glycogen, leaving a liquid which filters well.

B. Rabbit. The method was the same as that described above, a dozen rabbits being used for each preparation. They were previously fed on large quantities of carrot.

C.   Oyster.   A gross of oysters (1500 grms.) were worked up by Pflüger's method and yielded 120 grms. purified glycogen.

D.   Yeast.   The glycogen was separated and purified from yeast gum by the method recently described by Harden and Young [1912] with the exception that yeast juice was employed as the starting material.   Large quantities of yeast juice were boiled, filtered and the filtrate precipitated with alcohol.   The precipitate was then heated on the boiling water bath with 60 per cent. caustic potash to remove protein, the solution diluted and again precipitated with alcohol.   After thorough washing with 50 per cent. alcohol and reprecipitation, the glycogen was purified from yeast gum by saturation of the solution with ammonium sulphate, which precipitates the glycogen but not the gum.   This process was repeated three times and the glycogen then dialysed to remove the ammonium sulphate.   The solution was then again precipitated with alcohol and the further purification carried out as in the final stages described above.   The preparation is extremely tedious and the yield very variable according to the history of the yeast employed.

The ash content of the four specimens was as follows.   Dog glycogen, 0·38 per cent.   Oyster, 0·2 per cent.   Rabbit, 0·51 per cent.   Yeast, 0·87 per cent.   The reaction of the two latter was faintly alkaline, while the dog and oyster preparations both yielded slightly acid solutions.

PREPARATION OF ENZYME.

100 grms. of pig's pancreas were minced and well ground up with half the weight of sand.   Sufficient kieselguhr was added to form a fairly stiff mass which was then pressed out in a hydraulic press.   The extract obtained in this way after filtration yielded a clear yellow-brown liquid which was extremely active.   It was usually diluted to five or ten times its volume before use.

EXPERIMENTAL METHODS.

The course of the hydrolysis can be followed in several ways: (i) estimations of the residual glycogen, (ii) determinations of the optical activity, and (iii) estimations of the reducing power of the solution after known intervals of time.   The first of these methods however is unsatisfactory owing to the fact that some of the higher dextrins are also precipitated by alcohol as well as the glycogen, for a precipitate is still obtained in this way some time after the disappearance of the iodine reaction.   The optical activity of the solution also is due to a complex mixture of substances, the nature of which has not been thoroughly worked out and hence in the majority of the following experiments, the hydrolysis has been

followed by estimations of the reducing power alone, though this method is not altogether free from the same objection. The latter were carried out by Bertrand's method and the reducing power calculated as maltose, preliminary experiments having shewn that under the conditions employed very little if any hydrolysis of the maltose into glucose took place. The reducing power of a maltose solution of known strength was first determined and from the results obtained, a curve constructed from which the maltose corresponding to any found weight of copper could be read off.

The experiments were all carried out in flasks of Jena glass which were used for no other purposes. In most cases toluene was added to the solutions to prevent bacterial contamination.

Hydrolysis of Glycogen by an Extract of Pig's Pancreas.

To study the general course of the hydrolysis, 500 c.c. of a 1 per cent. solution of oyster glycogen were incubated with 2·5 c.c. of a pancreas extract with the addition of 5 c.c. toluene as antiseptic. The glycogen and enzyme solutions were each brought to the temperature of the bath (37°) before mixing and then after known intervals of time, samples of the liquid were removed and the following estimations made.

A. Reducing power.

B. Matter precipitable by alcohol (glycogen and higher dextrins). The precipitation was carried out by adding two volumes of alcohol. At the same time the reaction of the solution towards iodine was observed.

The results are collected in the following table.

TABLE I.

Hydrolysis of Glycogen by Pancreatic Diastase.

Time	Duration of action	Wt. of ppt. thrown down by 2 vols. alcohol	Maltose	Iodine reaction	Percentage hydrolysis
12.30	0	4·702 grms.	—	Dark red brown	0
12.35	5 mins.	—	0·223 grm.	Red brown	4·45
12.45	15 ,,	3·49 ,,	0·664 ,,	,,	13·32
1.00	30 ,,	2·74 ,,	1·156 grms.	,,	23·2
1.15	45 ,,	2·30 ,,	1·471 ,,	Pale red brown	29·5
1.30	60 ,,	1·83 ,,	1·714 ,,	Very faint	34·4
2.0	90 ,,	1·48 ,,	2·003 ,,	None	40·2
2.30	2 hrs.	1·20 ,,	2·145 ,,	—	43·0
3.30	3 ,,	—	2·292 ,,	—	46·0
4.30	4 ,,	0·65 grm.	2·362 ,,	—	47·4
5.30	5 ,,	—	2·434 ,,	—	48·8
9.30	9 ,,	Estimation spoilt	2·550 ,,	—	51·2

In Fig. 1 the results are shewn graphically, curve A representing the degree of hydrolysis and curve B the residual glycogen and dextrins precipitable by two volumes of alcohol. It will be seen that the action commences at a high initial velocity and proceeds for a very brief period in a linear direction. By the time the iodine reaction has disappeared, the rate has slowed down considerably and finally a long period of extremely slow hydrolysis follows. It would appear that the rapid initial stage is chiefly concerned with the conversion of the glycogen into dextrins with simultaneous
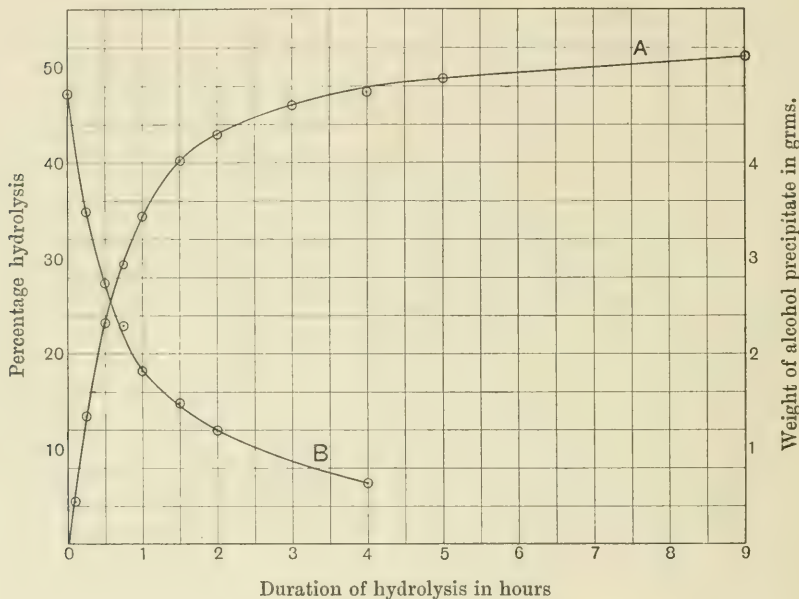
Fig. 1.

production of sugar. These dextrins, which are very resistant to further hydrolysis, are then slowly broken down to sugar. It is obvious however that in the above experiment this second stage would never have reached completion and this seems to be the general rule unless very high enzyme concentrations are employed. The iodine reaction disappeared when about 40 per cent. of the glycogen had been completely hydrolysed to maltose. The action in short closely resembles the hydrolysis of starch but is slower and less complete.

INFLUENCE OF TEMPERATURE.

A series of flasks each containing 50 c.c. of 1 per cent. glycogen were incubated with 0·5 c.c. enzyme solution, at temperatures varying from 25 to 50°. Thirty minutes after the addition of the enzyme, the action was stopped by adding 5 c.c. of N . KOH, the liquid was at once cooled and the volume made up to 100 c.c. The reducing power of 20 c.c. of this dilution was then estimated. The results are shewn below in Table II and graphically in Fig. 2. For the sake of comparison the results of a starch hydrolysis with the same enzyme preparation are given also.

TABLE II.

Influence of Temperature.

Temperature of incubation	A. Glycogen		B. Starch	
	Total maltose	Percentage hydrolysis	Total maltose	Percentage hydrolysis
25·5°	0·1070	20·2	—	—
27·5°	—	—	0·1310	24·7
34°	0·1212	22·9	—	—
37°	0·1375	25·9	0·1545	29·2
39·5°	0·1330	25·1	—	—
42°	0·1310	24·7	0·1700	32·1
46°	0·1260	23·7	0·1895	35·7
50·5°	0·1190	22·5	0·1760	33·2

A. Glycogen.
B. Soluble Starch.
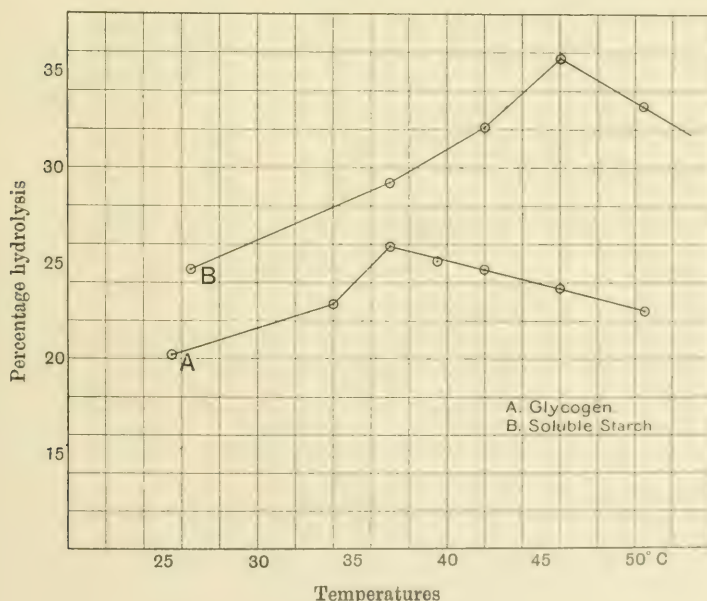
Temperatures

Fig. 2.

Hence with the enzyme employed in these experiments, the optimum temperature for glycogen hydrolysis was about 37°C. and this value was unchanged by substituting different preparations of glycogen. For starch hydrolysis on the other hand the optimum temperature found with the same enzyme solution was 46°C. This result therefore lends some support to the hypothesis that the two actions are brought about by two distinct enzymes.

INFLUENCE OF ENZYME CONCENTRATION.

Three dilutions of the enzyme solution were prepared and 0·5 c.c. of each added to a series of flasks each containing 50 c.c. of a 1 per cent. glycogen solution (oyster) + 1 c.c. toluene and previously brought up to 37°. Altogether three flasks were used with each dilution.

After 5, 15, and 25 minutes' incubation one flask of each series was removed from the bath and the action stopped by the addition of 5 c.c. N. alkali. The reducing power of each solution was then determined and the results are shewn in Table III below.

TABLE III.

Influence of Enzyme Concentration.

Each flask contained 50 c.c. 1 per cent. glycogen + 1 c.c. toluene + 0·5 c.c. enzyme.

Exp.	Dilution of enzyme solution	Reduction after time given, mgrms. Cu from 20 c.c. liquid		
		5 mins.	15 mins.	25 mins.
A	1/10	7	21·5	32·0
B	1/20	3·35	10·0	—
C	1/40	1·7	5·2	9·0

These results shew very clearly two things. (i) Under the above conditions the rate of hydrolysis is directly proportional to the concentration of enzyme, and (ii) the action is a linear one, the reduction after 15 minutes' being almost exactly three times that after five minutes' incubation and so on. These results only obtain however when very dilute enzyme preparations are used and during the initial part of the experiment—in other words while there is a considerable excess of unchanged glycogen. Similar results have been obtained by Evans [1912] in the hydrolysis of starch by saliva.

When stronger preparations of enzyme are employed the above relations can only be observed for a very brief period of the hydrolysis, but another point now becomes more noticeable. With dilute enzyme preparations, the action comes to an end long before all the glycogen has been completely

converted into maltose. This is apparent in the results quoted in Table I where it will be noticed that after nine hours there was only a 51 per cent. hydrolysis and the action had practically come to an end. When however higher concentrations of enzyme are employed, the end point of the reaction is moved very much nearer to complete hydrolysis as will be seen from the experiments described below.

A series of flasks was incubated at 37°C. containing the following solutions :

A. 100 c.c. 1 per cent. glycogen solution + 4·9 c.c. H_2O + 0·1 c.c. enzyme + 1 c.c. toluene.

B. 100 c.c. 1 per cent. glycogen solution + 4·5 c.c. H_2O + 0·5 c.c. enzyme + 1 c.c. toluene.

C. 100 c.c. 1 per cent. glycogen solution + 4·0 c.c. H_2O + 1·0 c.c. enzyme + 1 c.c. toluene.

D. 100 c.c. 1 per cent. glycogen solution + 0 c.c. H_2O + 5·0 c.c. enzyme + 1 c.c. toluene.

Duration of hydrolysis in hours
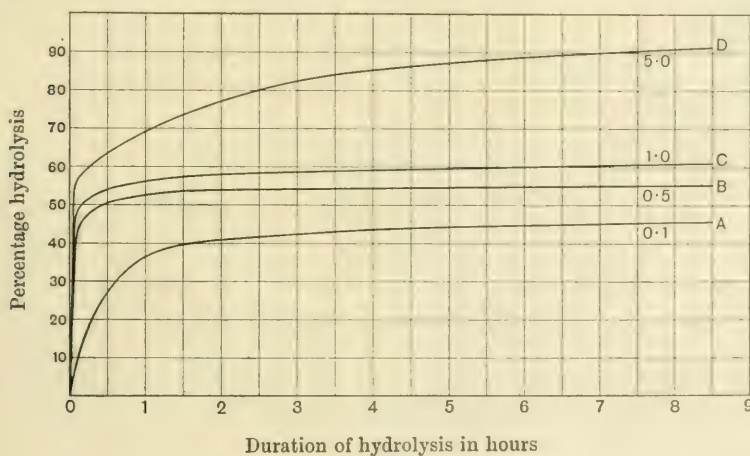
Fig. 3.

The total volume in each case was the same, the only difference being the concentration of enzyme. After $8\frac{1}{2}$ hours the action was almost over in all four solutions and the amount of hydrolysis which had taken place was as follows :

A, 45·6 per cent. B, 55·1 per cent. C, 60·7 per cent. D, 91·3 per cent. The complete course of the hydrolysis in each case is shewn in Fig. 3.

Very similar results were obtained by Philoche [1908] with taka diastase and it is not quite clear what interpretation is to be placed upon them, for, as

will be seen, there appears to be no direct relationship between the concentration of enzyme and the final degree of hydrolysis. The influence of the products formed during the hydrolysis may be partially responsible but judging from experiments described later, it is unlikely that this is the sole cause. The incompleteness of the hydrolysis cannot be ascribed to the destruction of the enzyme, for the latter after 20 hours' incubation rapidly attacked a second quantity of glycogen.

INFLUENCE OF GLYCOGEN CONCENTRATION.

Unless very low concentrations are used—less than 0·5 per cent. with the strength of enzyme I have generally employed—the *initial* rate of hydrolysis is practically unchanged by increasing the glycogen concentration. In the weaker solution, however, the rate naturally begins to fall at an earlier period owing to the glycogen being more rapidly exhausted. These points are illustrated by the results shewn in Table IV.

TABLE IV.

Influence of Glycogen concentration.

Time after addition of enzyme	Reduction, mgrms. Cu from 20 c.c. liquid		
	A. 1 % glycogen	B. 2 % glycogen	
5 mins.	9·0	9·0 }	Rates equal.
15 ,,	26·8	27·0 }	
30 ,,	45	52	Rate in A falling, B practically unchanged.
1 hr.	66	89 }	Both rates falling, but more rapidly in A.
4 hrs.	92	147 }	
20 ,,	108	188	Action at an end in both.
% hydrolysis	49·6	43·5	

INFLUENCE OF PRODUCTS.

As previously stated, unless high concentrations of enzyme are employed the hydrolysis is not complete. In order to see whether this was due to the influence of the products the following experiments were carried out.

Influence of Maltose. In a preliminary experiment the action had stopped when about 50 per cent. of the glycogen had been hydrolysed to maltose, i.e. when the solution contained rather over 0·5 per cent. of maltose. This

amount of maltose was therefore added to one flask at the beginning of the experiment and the rate of hydrolysis compared with one containing no sugar at the beginning. The composition of the two solutions was as follows:

Flask A. 100 c.c. 1 per cent. glycogen + 1 c.c. toluene + 0·5 c.c. enzyme.

Flask B. 100 c.c. 1 per cent. glycogen solution containing 0·5 grm. maltose + 1 c.c. toluene + 0·5 c.c. enzyme.

TABLE V.

Influence of Maltose.

Time after addition of enzyme	A. Glycogen alone	B. Glycogen + maltose		
	Mgrms. Cu from 20 c.c.	Mgrms. Cu from 20 c.c.	Cu due to added maltose	Cu due to maltose formed
15 mins.	27	136	108	28
30 ,,	46	154	,,	46
1 hr.	66	173	,,	65
2 hrs.	80	188	,,	80
4 ,,	92	199	,,	91
8 ,,	100	207	,,	99
20 ,,	108	212	,,	104
% hydrolysis	49·6			47·6

It will be seen from these results that the rate of hydrolysis was practically unchanged by the maltose added and the final degree of hydrolysis lowered by only two per cent. The dextrins formed during the course of the reaction, however, seemed to have some retarding influence, this being illustrated by the following experiment.

Influence of Dextrin. 100 c.c. of 2 per cent. glycogen were incubated at 37° with 0·5 c.c. enzyme until the solution no longer gave the iodine reaction. The action was then stopped by heating the flask in a boiling water-bath. The solution thus obtained was free from glycogen and in addition to the dextrins contained about 0·9 per cent. of maltose. It is referred to below as "products of hydrolysis." The following mixtures were then made up and incubated at 37°.

A. 50 c.c. 2 per cent. glycogen + 50 c.c. "products of hydrolysis" + 1 c.c. toluene + 0·5 c.c. enzyme.

B. 50 c.c. 2 per cent. glycogen + 50 c.c. H_2O + 1 c.c. toluene + 0·5 c.c. enzyme.

Incubation was continued for six hours, the reducing power of the two solutions being estimated from time to time. The results are collected in Table VI.

TABLE VI.

Influence of Products of Hydrolysis.

Time after addition of enzyme	B. Glycogen alone	A. Glycogen + products		
	Reduction, mgrms. Cu from 20 c.c. liquid	Reduction, mgrms. Cu from 20 c.c. liquid	Cu due to sugar present at start	Cu due to maltose formed
15 mins.	26	110	84	26
30 ,,	45	128	,,	44
1 hr.	66	149	,,	65
2 hrs.	81	159	,,	75
4 ,,	90	166	,,	82
6 ,,	97	170	,,	86
% hydrolysis	44·6			39·3

In this case although there was again no difference in the initial rates, after one hour the hydrolysis in A began to lag behind that in B and after six hours only 39·3 per cent. of the glycogen in A had been hydrolysed as against 44·6 per cent. in B. Moreover in A the action had almost stopped while in B it was still proceeding at a fair rate. We have here then a marked hindering action which must apparently be due to the dextrin-like substances formed during the hydrolysis.

INFLUENCE OF ACIDITY OR ALKALINITY.

The ash almost always present in samples of glycogen consists to a large extent of phosphates and according to the nature of these phosphates the solution may have either an acid or alkaline reaction. Before comparing different samples of glycogen it was therefore necessary to determine to what extent this difference in reaction would influence the rate of hydrolysis. It is obvious moreover that the optimum dose of acid will vary according to the source and proportion of the enzyme used, for the latter as well as the solution acted on may contain substances capable of neutralising some of the acid. On the other hand the optimum hydrogen ion concentration is a constant independent of these factors. In the latter case we obtain a measurement of the true optimum reaction of the solution, while in the former we have only an apparent value, the degree of accuracy of which will depend on the amount and nature of the salts present in the solution. Thus Sörensen [1909] working with three different preparations of invertin found the optimal dose of sulphuric acid (apparent acidity) to be very different for the three preparations but the optimum hydrogen ion concentration (true

acidity) was the same in each case. Hence in all the following experiments the reaction of the solution has been determined by measurements of the hydrogen ion concentration.

At first attempts were made to do this by means of the colorimetric method described by Sörensen. The strong opalescence of the glycogen solutions however rendered this method impracticable and hence the electrical method has been used throughout. The apparatus employed was that of Michaelis and Rona [1909] which is especially suitable since only small quantities of solution are required. In this method, the electrodes are kept in a still atmosphere of hydrogen and connection is made between the two cells by means of a tape soaked in a solution of potassium chloride. By making two determinations using different concentrations of KCl, e.g. 1·75 N. and 3·5 N., the contact potential due to the salt can be calculated and corrected for and, as equilibrium is established almost immediately, the method is both rapid and convenient. The determinations were all made at 37°, the temperature at which the hydrolyses were carried out, the two operations being conducted in the same thermostat. In each case the standard solution against which the glycogen solutions were compared was N/10 HCl and from the value of the E.M.F. thus set up and determined, the hydrogen ion concentration was calculated in the usual way[1].

The latter is denoted by the sign p_H, this being the logarithm of the reciprocal value of the factor of normality. p_H being known, the hydroxyl ion concentration can be calculated from the dissociation constant of water. By graphical interpolation from a series of determinations by Lundén [1907], the value at 37° has been taken as $2·45 \times 10^{-14} = 10^{-13·61}$. Hence in a N/100 solution of hydrogen ions where $p_H = 2$ the hydroxyl ions will have a concentration at 37° of $10^{-11·61}$ and so on. In the same way at absolute neutrality the concentration of both ions will be $10^{-6·8}$.

DETERMINATION OF OPTIMUM REACTION.

To a series of flasks each containing 100 c.c. of a 1 per cent. glycogen solution, were added varying amounts of N/100 NaOH or H_2SO_4. The total volume of the solutions, however, was kept the same in every case. Each flask was then in turn placed in the bath at 37° and when the solution had acquired that temperature 0·5 c.c. of a dilute enzyme preparation was added.

[1] A full account will be found in Sörensen's paper [1909] where he also discusses the possible sources of error in the method.

After thorough mixing, a sample was removed and the hydrogen ion concentration at once determined. Thirty minutes after the addition of the enzyme, a second sample was taken, the action stopped by the addition of a small quantity of N. alkali and the reducing power estimated. In this way the true reaction and the degree of hydrolysis was determined in each case. This process was repeated with all four preparations of glycogen. In these experiments no toluene or other antiseptic was used, Sörensen having found that these substances interfere somewhat in the hydrogen ion determinations, equilibrium not being so readily established. As the duration of each experiment, however, was only 30 minutes it is highly improbable that this in any way influenced the results. These are collected in Table VII and also shewn graphically in Fig. 4.

TABLE VII.

Influence of hydrogen ion concentration on the rate of hydrolysis.

Experiment	Source of glycogen	C.c. of N/100 acid or alkali added		E.M.F. volts	p_H	Reduction, mgrms. maltose in 20 c.c.	% hydrolysis in 30 mins.
1	Dog liver	3·0	H_2SO_4	0·2055	4·43	7·37	3·7
2	,,	1·0	,,	0·2306	4·84	33·5	16·8
3	,,	0·5	,,	0·2754	5·57	52·9	26·6
4	,,	0	,,	0·2940	5·87	59·0	29·6
5	,,	0·25 NaOH		0·3018	6·00	61·4	30·8
6	,,	0·5	,,	0·3217	6·32	60·3	30·3
7	,,	1·0	,,	0·3410	6·63	54·0	27·1
8	,,	3·0	,,	0·3755	7·19	37·1	18·6
9	,,	6·0	,,	0·4078	7·72	12·1	6·1
14	Oyster	0·5	H_2SO_4	0·2260	4·76	28·0	14·1
15	,,	0	,,	0·2730	5·53	40·4	20·3
16	,,	0·5 NaOH		0·2870	5·76	44·2	22·2
17	,,	1·0	,,	0·3019	6·00	44·4	22·3
18	,,	3·0	,,	0·3322	6·49	32·8	16·5
19	,,	6·0	,,	0·3980	7·56	10·7	5·4
22	Rabbit liver	6·0	H_2SO_4	0·2492	5·14	4·8	2·4
23	,,	5·0	,,	0·2750	5·56	35·6	17·9
24	,,	4·75	,,	0·2850	5·72	45·2	22·7
25	,,	4·5	,,	0·3010	5·97	47·5	23·9
26	,,	4·0	,,	0·3180	6·26	47·0	23·6
27	,,	3·0	,,	0·3412	6·64	42·3	21·3
28	,,	0	,,	0·3818	7·30	24·9	12·5
29	Yeast	6·0	H_2SO_4	0·2748	5·55	25·5	12·8
30	,,	5·0	,,	0·2940	5·87	39·5	19·8
31	,,	3·0	,,	0·3120	6·16	40·0	20·1
32	,,	1·5	,,	0·3245	6·36	39·5	19·8
33	,,	0	,,	0·3580	6·91	27·1	13·6

The results shewn above indicate that the value obtained for the optimum hydrogen ion concentration was practically the same with each preparation of glycogen, that from oyster however giving a slightly lower figure than the other three. The optimum reaction therefore for the enzyme I have used was just on the acid side of the neutral point, the hydrogen ion concentration corresponding to an acidity of about $N/10^6$. Alteration of the reaction in either direction produced a considerable reduction in the rate of hydrolysis especially in the case of the dog and oyster glycogens. Hence in comparing the rate of hydrolysis of different glycogen preparations it is essential to see that the reactions of the solutions are as nearly the same as possible, if the results are to be at all reliable.
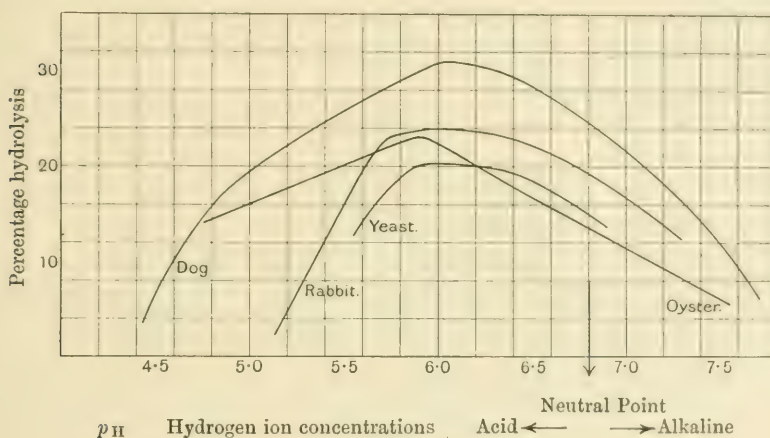
Fig. 4.

COMPARISON OF GLYCOGENS.

1 per cent. solutions of the four kinds of glycogen were prepared and sufficient $N/100$ acid or alkali added so that each solution after the addition of the enzyme was as nearly as possible at the optimum reaction. The amount to be added was estimated from the results obtained in the experiments last described, the total volume was the same in each case. Immediately after the addition of the enzyme, the hydrogen ion concentration was determined to make sure that the reaction was really correct and then after 30 minutes' hydrolysis, the action was stopped by the addition of alkali and the reducing power estimated. Both the hydrolysis and the hydrogen ion determination were carried out at 37° and no toluene was used for the reason already stated. The results are shewn in Table VIII.

TABLE VIII.

Comparison of Glycogens.   I.

Source of glycogen	E. M. F. of solution against N/10 HCl at 37° C.	p_H	Mgrms. maltose per 20 c.c. after 30 mins.	Percentage hydrolysis after 30 mins.	Relative rates
Dog	0·2970 volts	5·92	46·10	23·05	100
Rabbit	0·2986 ,,	5·94	43·25	21·62	93·82
Oyster	0·2956 ,,	5·90	41·0	20·5	88·9
Yeast	0·3014 ,,	5·99	39·5	19·7	85·7

COMPOSITION OF SOLUTIONS.

A.   Dog glycogen. 100 c.c. 1 per cent. glycogen + 0·25 c.c. N/100 NaOH + 4·5 c.c. H_2O + 0·5 c.c. enzyme.

B.   Oyster. 100 c.c. 1 per cent. glycogen + 1·0 c.c. N/100 NaOH + 4·0 c.c. H_2O + 0·5 c.c. enzyme.

C.   Rabbit. 100 c.c. 1 per cent. glycogen + 4·5 c.c. N/100 H_2SO_4 + 0·5 c.c. H_2O + 0·5 c.c. enzyme.

D.   Yeast. 100 c.c. 1 per cent. glycogen + 4·25 c.c. N/100 H_2SO_4 + 0·75 c.c. H_2O + 0·5 c.c. enzyme.

There is thus quite a marked difference in the rate of hydrolysis of the four preparations of glycogen examined, the least difference being between those from oyster and yeast. A second experiment gave very similar results which are shewn in Table IX.

TABLE IX.

Comparison of Glycogens.   II.

Source of glycogen	E. M. F. of solution against N/10 HCl at 37° C.	p_H	Mgrms. maltose per 20 c.c. after 30 mins.	Percentage hydrolysis	Relative rates
Dog	0·3046 volts	6·0	48·9	24·45	100
Rabbit	0·2950 ,,	5·9	46·1	23·05	94·3
Oyster	0·2901 ,,	5·8	42·4	21·2	86·7 *
Yeast	0·3112 ,,	6·1	40·45	20·2	83 †

     * Solution rather too acid.   † Solution rather too alkaline.

OPALESCENCE OF GLYCOGEN SOLUTIONS.

A 0·2 per cent. solution was made up of each of the four preparations of glycogen and the opalescence of these compared. This was carried out in Nessler glasses, the volume of solution being adjusted until the opalescence appeared to be the same in each case. The comparison is not easy and the experimental error is therefore large but the solution of rabbit glycogen was

markedly the most opalescent of the four examined, oyster and dog glycogen being about equal and yeast less opalescent than any. The actual results obtained were as follows:

TABLE X.

Glycogen	Volumes of 0·2 solutions having equal opalescence	Relative opalescence
Rabbit	11 c.c.	4·5
Dog	30 ,,	1·7
Oyster	33 ,,	1·5
Yeast	50 ,,	1

COLOURATION WITH IODINE.

To 10 c.c. of a 0·2 per cent. solution of each of the glycogen preparations was added 1 c.c. of a 1 per cent. solution of iodine. The volume in each case was then made up to 50 c.c. and the colours compared in Nessler glasses with the following results:

TABLE XI.

Glycogen	Volumes of solutions giving equal depth of colour	Relative strength of colouration
Rabbit	10·5 c.c.	4·7
Dog	19 ,,	2·6
Yeast	32 ,,	1·5
Oyster	50 ,,	1

Thus both as regards opalescence and the colouration with iodine there is a distinct difference between the four glycogen solutions.

SUMMARY AND CONCLUSIONS.

The conditions under which glycogen is hydrolysed by a pancreatic extract (pig) have been studied with the following results:

1. The hydrolysis at first proceeds with great rapidity, the glycogen being quickly converted into dextrins with simultaneous production of maltose. The further breaking down of these dextrins takes place with extreme slowness and is as a rule incomplete.

2. The optimum temperature for glycogen hydrolysis of the enzyme employed in these experiments was 37°. Below this there is a rapid fall in the rate, between 37° and 50° this is less marked. With starch the optimum temperature for the same enzyme preparation was 46°.

3. While excess of glycogen is present, the rate of change is directly proportional to the enzyme concentration and the action is a linear one.

4. With low enzyme concentrations, the action is by no means complete; the degree of hydrolysis, however, rises with increasing concentration of enzyme though not in the same ratio.

5. The concentration of the glycogen solution has little influence on the initial rate of hydrolysis unless very low concentrations (less than 0·5 per cent. in my experiments) are employed.

6. The action is hindered to a small extent by the products of hydrolysis, the dextrins probably being the interfering substances.

7. The action is favoured by the presence of small traces of acid, the optimum reaction determined with several preparations of glycogen being a hydrogen ion concentration of 10^{-6} (at neutrality this value is $10^{-6.8}$ at $37°$).

8. Samples of glycogen prepared from (i) dog, (ii) rabbit, (iii) oyster, and (iv) yeast are hydrolysed at different rates when compared at the optimum hydrogen ion concentration. Taking dog glycogen as 100, the relative rates of hydrolysis found are, rabbit 94, oyster 88, and yeast 84.

9. The degree of opalescence and the colouration with iodine exhibited by glycogen solutions vary with the source from which the glycogen has been obtained.

10. In view of the above points of difference it is probable that the four preparations of glycogen examined are not identical in constitution, though it is possible these differences might be caused by variations in the colloidal state of the four glycogen solutions. If, however, the glycogens are distinct, the diastatic enzymes obtained from different animals should also be specific and experiments are in progress to test this point.

REFERENCES.

Bertrand (1906), *Bull. Soc. Chim.* **35**, 1285.
Clautriau (1895), 'Etude chemique du glycogène,' *Mem. Couronnes, Acad. Roy. Belg.* **53**.
Cremer (1894), *München. Med. Wochensch.* **41**, 525.
Evans (1912), *J. Physiol.* **44**, 191.
Ford (1904), *J. Soc. Chem. Ind.* **23**, 414.
Harden and Young (1902), *J. Chem. Soc.* **81**, 1225.
Harden and Young (1912), *J. Chem. Soc.* **101**, 1928.
Lundén (1907), *J. Chim. Phys.* **5**. 574.
Michaelis and Rona (1909), *Biochem. Zeitsch.* **18**, 318.
Pflüger (1905), "*Das Glykogen*," Bonn.
Philoche (1908), *J. Chim. Phys.* **6**, 212, 355.
Sörensen (1909–1910), *Compt. rend. Carlsberg*, **8**, 1.

IV. THE METABOLISM OF ORGANIC PHOSPHORUS COMPOUNDS. THEIR HYDROLYSIS BY THE ACTION OF ENZYMES.

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INTRODUCTION.

Our knowledge concerning the composition of the organic phosphorus compounds occurring in both plants and animals is still very rudimentary. Five groups of organic phosphorus compounds occur in nature :—

1. *Phospholipines*, of which lecithin is the principal example, and in which the phosphorus is regarded as being in ester combination with glycerol as glycerophosphoric acid ;

2. *Phytin*, in which phosphoric acid is in combination with inositol as inositol-phosphoric acid, the compound itself being regarded as the calcium magnesium salt ;

3. *Hexosephosphate*, in which a hexose is combined with phosphoric acid ;

4. *Nucleic acid*, in which again a carbohydrate (hexose or pentose) is combined with phosphoric acid, and attached to the carbohydrate is a purine base ;

5. *Phosphoprotein*, in which phosphoric acid is in an unknown form of combination, presumably with one of the amino-acids composing the protein molecule.

The phospholipines occur chiefly in animals and only to a small extent in plants ; phytin or phytic acid has only been found in plants ; hexosephosphate is a product formed in alcoholic fermentation by yeast ; nucleic acid is said to be the chief constituent of the cell nuclei in both plants and animals ; phosphoprotein is present only in the animal kingdom and forms the chief protein foodstuff of embryo birds and suckling mammals.

Considering how little is known as to the chemical composition of these

compounds it is not to be wondered at that the knowledge of their metabolism is vague. Most investigators will agree that plants can synthesise organic phosphorus compounds from inorganic phosphates and that they are also able to carry out the reverse process, but how these changes are effected, or by what enzymes they are caused, is practically unknown.

Most of the experiments on phosphorus metabolism in animals have been feeding experiments in which animals have been fed with organic and inorganic phosphorus compounds and the total intake and output of phosphorus estimated, together with that of nitrogen in most cases. The conclusions which were drawn from the general condition of the animal at the end of the time, and from the effect of the phosphorus compounds upon the nitrogen metabolism, were summarised by F. C. Cook in 1909. They led to the belief that organic phosphorus compounds are directly assimilated and are preferable to inorganic phosphates as the source of phosphorus for animals. Against this belief the recent work of Fingerling [1912; 1, 2] shows that the animal organism can synthesise organic phosphorus compounds from inorganic phosphates. He fed ducks for long periods on food containing only organic phosphorus, or only inorganic phosphorus, as the source of phosphorus. Both sets of ducks laid approximately the same number of eggs and the lecithin and nuclein content of the eggs was about the same in either case. He also analysed the milk of goats which were fed with organic and inorganic phosphorus in the same way as he fed the ducks. In both cases the composition of the milk was the same. Gregersen [1911] has also found that the organic phosphorus compounds can be built up in the animal body from inorganic phosphate, thus confirming the work of Fingerling.

Both organic and inorganic phosphorus compounds can therefore be utilised for the formation of the organic phosphorus compounds in animals; the feeding experiments with organic phosphorus compounds do not show in what form the phosphorus is assimilated, i.e. whether it is absorbed in an organic form or whether it is first hydrolysed and then resynthesised. Phosphorus is excreted in the urine of animals almost entirely as inorganic P_2O_5, whether the intake be organic or inorganic; only a small quantity is excreted in an organic form [Mathison, 1909; Plimmer, Dick and Lieb, 1909; and Gregersen, 1907], and the composition of this organic phosphorus compound is unknown although it is stated to be glycerophosphoric acid. The phosphorus in the faeces is also mainly inorganic P_2O_5. These facts point to the hydrolysis or decomposition of organic phosphorus compounds in the animal organism.

It seemed to me that the only possible means by which the metabolism and assimilation of the natural organic phosphorus compounds could be studied was to ascertain if these compounds were hydrolysed by the enzymes of the digestive tract. The action of the enzymes of the pancreas, intestine and liver upon the various organic phosphorus compounds has therefore been investigated and in addition the action of some plant enzymes has been included so as to obtain a complete and comparative survey of the whole series of organic phosphorus compounds.

I. Glycerophosphoric acid.

The literature on phosphorus metabolism in plants contains no statement as to the hydrolysis or synthesis of glycerophosphoric acid or of lecithin, and until quite recently the knowledge of the metabolism of these substances in animals was equally indefinite. The experiments made by Marfori [1905], Bülow [1894], Mathison [1909], Plimmer, Dick and Lieb [1909] and others had shown that glycerophosphoric acid was completely absorbed when introduced into the body either by the mouth or subcutaneously, and that the phosphorus appeared in the urine as inorganic phosphate and not as glycerophosphoric acid. At the time when these investigations were made[1] no one had tried if the organs of the animal body were able to hydrolyse glycerophosphoric acid and convert it into glycerol and phosphoric acid. It was generally assumed that glycerophosphoric acid was either assimilated as such, or that, since it is an ester, it was hydrolysed like the fats by the lipase in the liver, pancreas and other organs. In 1912 Grosser and Husler [1912] first published experiments which proved that an enzyme (glycerophosphatase) able to hydrolyse glycerophosphoric acid was present in certain animal tissues. They found that the kidney and intestine could completely hydrolyse glycerophosphoric acid, that the lung hydrolysed 62 per cent. and the liver 16 per cent. The spleen, blood and muscle did not hydrolyse glycerophosphoric acid.

The fact that glycerophosphatase is present in the intestine, kidney and lung but not in the pancreas and liver suggests that the hydrolysis is not effected by lipase since the pancreas and liver are the chief animal tissues which contain lipase, whereas the other organs mentioned only contain small amounts of this enzyme.

An enzyme which can hydrolyse glycerophosphoric acid is also present in plants, especially in castor oil seeds. Like the lipase in these seeds it is only

[1] The results of my investigations upon the metabolism of glycerophosphoric acid were communicated to the Biochemical Club at their meeting in July 1911.

liberated from a precursor by the action of dilute acids, but unlike the lipase it is soluble in water [Connstein, Hoyer and Wartenberg, 1902; Armstrong and Ormerod, 1906; Tanaka, 1910]. The action of the two enzymes is thus distinct although it has been impossible to prove this absolutely, as after repeated extraction with water the final extract always contained some glycerophosphatase.

Extracts of yeast and of bran, which contain the enzymes hexosephosphatase [Harden and Young, 1908] and phytase [Suzuki and Takaishi, 1907] respectively, are also able to hydrolyse glycerophosphoric acid.

EXPERIMENTAL.

The general method for determining the action of extracts of various tissues upon glycerophosphoric acid has been to mix a known volume of the extract with either a known volume of a solution of glycerophosphoric acid previously neutralised with soda, or with a solution of sodium glycerophosphate; to withdraw a sample of known volume immediately after mixing and after subsequent intervals of time; and to estimate, after filtering if necessary, the inorganic phosphate in these samples by precipitation with ammonium magnesium citrate in the presence of ammonia, and conversion into magnesium pyrophosphate. By the use of magnesium citrate instead of magnesia mixture the precipitation of organic matter with the ammonium magnesium phosphate is avoided, as was shown by Plimmer and Bayliss in 1906. The total phosphorus content of the mixture was estimated in another portion by Neumann's method as modified by Plimmer and Bayliss [1906]. All results were then calculated for the same volume. In some cases a control experiment consisting of the same volumes of water and of extract was carried out simultaneously. Toluene was added as an antiseptic and the mixtures were kept at 37° C. after the removal of the first sample.

The commercial preparations of glycerophosphoric acid and of sodium glycerophosphate, which are synthetically prepared, were employed. The synthetical compound except for its optical activity is apparently identical with the natural compound obtained from lecithin. Both products seem to be mixtures [Willstätter and Lüdecke, 1904; Power and Tutin, 1905; Tutin and Hann, 1906].

It was originally supposed that the glycerophosphoric acid, since it is an ester, would be hydrolysed by the lipase of the pancreas and other tissues: for this reason the preparations and extracts of tissues used have been purposely made by the usual methods for obtaining an active lipase.

The action of the pancreas was investigated with :

1. The residue from an aqueous extract of trypsin (Fairchild); such residues have been shown by Dietz [1907] to be very strongly lipoclastic in their action. The residue was suspended in a solution of sodium glycerophosphate and the inorganic P_2O_5 was estimated after filtering off and washing the precipitate.

2. A glycerol extract of pig's pancreas prepared by extracting the organ for 24–36 hours with ten times its bulk of a mixture consisting of 90 parts of glycerol and 10 parts of 1 per cent. sodium carbonate, and then straining through muslin. In one experiment this extract was used in aqueous solution, in another in about 50 per cent. glycerol solution. The mixture containing the extract of sodium glycerophosphate was then made just alkaline to phenolphthalein and maintained at 37° C. in the presence of toluene. The samples from the glycerol solution were diluted with water and filtered, and inorganic P_2O_5 was estimated in the filtrate and washings.

3. Fresh pancreatic juice which is known to contain an active lipase. The pancreatic juice in one experiment was kindly collected for me by Prof. Starling, in another experiment by Prof. Bayliss; in the first experiment the juice was activated by enterokinase by adding a small amount of intestinal extract ; in the second experiment the juice was not activated.

The action of the liver was investigated with :

1. An extract of dog's liver made by the method of Loevenhart and Peirce [1906] for obtaining an active lipase.

2. An extract of calf's liver prepared according to the method of McCollum and Hart [1908] in their study of phytase in animal tissues. The samples removed at intervals were acidified with a drop of glacial acetic acid, and were boiled and filtered. The coagulum was thoroughly washed and inorganic P_2O_5 was estimated in the filtrate and washings.

The action of the small intestine was investigated by extracts made from the mucous membrane, which was ground up with sand and extracted for 24–48 hours with water containing toluene as antiseptic ; and then strained through lint or muslin. In the earlier experiments this extract which was milky in appearance was used directly ; the samples, which were removed at intervals, were acidified with acetic acid; the precipitate so formed was filtered off and thoroughly washed and the inorganic P_2O_5 estimated in the clear filtrate and washings. In the later experiments the aqueous extract of the mucous membrane was acidified with acetic acid, and filtered from the precipitate : the clear extract so obtained was found to be active, either when still acid, or after neutralising with sodium carbonate. These clear extracts

were used in all the later experiments with the other organic phosphorus compounds.

The action of the kidney and liver was investigated with extracts made by grinding the organ with sand, and extracting the mass with water, to which toluene was added, for 1–2 days. In the first experiment with the kidney the extract was acidified with acetic acid, the filtrate neutralised with sodium carbonate and used after again filtering. In the other experiments the milky extract was used; each sample was acidified and diluted with the same volumes of acetic acid and water. Inorganic phosphate was estimated in a measured volume of the clear filtrate.

A suspension of shelled castor oil seeds in dilute acetic acid was used for the detection of a glycerophosphatase, samples being removed and the inorganic P_2O_5 estimated in the filtrate and washings. A suspension was also employed when the seed was treated with acid, washed and the extracted residue tested for glycerophosphatase.

Extracts of castor oil seeds were prepared by treating the shelled seeds ground as finely as possible, sometimes after the fat had been previously removed by extraction with ether, with water or with decinormal acetic acid for 1–2 days in the proportion of 5–20 grams of seed and 100–300 c.c. of the acetic acid. A clear filtrate was obtained on filtration. These filtrates were used directly and in some experiments after neutralisation of the acid extract with sodium carbonate.

Preliminary experiments to ascertain if a glycerophosphatase were present in yeast were made with an aqueous extract of pressed yeast prepared in the presence of toluene and by a suspension of zymin; in the latter case the estimations of inorganic P_2O_5 were made in the filtrate and washings from the insoluble matter.

The later and all subsequent experiments with yeast enzymes were made with extracts of the commercial "zymin" of Schroder and Co. of Munich. These extracts were prepared by treating the powder with ten times its weight of water for 24–48 hours in the presence of toluene and then filtering. A pale brown solution resulted.

By simply covering wheat bran with five times its weight of water in the presence of toluene a clear and active extract was always obtained on filtering. The bran extract if kept without contact with air was of a pale brown colour, but when it was exposed to the air and filtered it became rapidly darker in colour.

The following are the analytical data:

Pancreas.

(1) Residue from 1·5 gm. trypsin (Fairchild) suspended in 500 c.c. water containing 5 gm. sodium glycerophosphate.

P₂O₅ in gm.

At commencement	0·0121
After 31 days	0·0115
Total 0·1712	

(2) 100 c.c. glycerol extract of pig's pancreas + 5 gm. sodium glycerophosphate in water. Mixture made alkaline to phenolphthalein and volume diluted with water to 500 c.c.

P₂O₅ in gm.

At commencement	0·0128
After 9 days	0·0135
Total 0·1775	

(3) 100 c.c. glycerol extract of pig's pancreas + 5 gm. sodium glycerophosphate dissolved in 200 c.c. glycerol + 200 c.c. water and mixture made alkaline to phenolphthalein.

P₂O₅ in gm.

At commencement	0·0120
After 8 days	0·0126
Total 0·1712	

(4) 20 c.c. glycerophosphoric acid diluted to 500 c.c. with water and neutralised with caustic soda + 20 c.c. pancreatic juice of dog + 4–5 drops intestinal juice. Total volume made up to 1000 c.c.

P₂O₅ in gm.

At commencement	0·0126	After 8 days	0·0759
After 1 day ...	0·0517	,, 10 ,,	0·0799
,, 2 days	0·0569	,, 13 ,,	0·0824
,, 3 ,,	0·0633	,, 20 ,,	0·0891
,, 5½ ,,	0·0723	,, 36 ,,	0·1110
	Total 0·2606		

(5) 2·5 gm. sodium glycerophosphate in water + 50 c.c. pancreatic juice of dog. Total volume made up to 500 c.c.

P₂O₅ in gm.

At commencement	0·0135
After 14 days	0·0124
Total 0·1661	

Liver.

(1) A. 100 c.c. extract of dog's liver by Loevenhart and Peirce's method + 400 c.c. H₂O. B. 100 c.c. same extract + 400 c.c. water containing 5 gm. sodium glycerophosphate.

P₂O₅ in gm.

	A	B
At commencement	0·0006	0·0147
After 1 day ...	0·0013	0·0165
,, 3 days ...	—	0·0157
,, 5 ,, ...	—	0·0122
Total	0·0177	0·1889

(2) A. 125 c.c. extract of calf's liver by McCollum and Hart's method + 125 c.c. water. B. 125 c.c. same extract + 125 c.c. water containing 2·5 gm. sodium glycerophosphate.

P₂O₅ in gm.

	A	B	Difference
At commencement ...	0·0232	0·0529	0·0297
After 5 days...	0·0339	0·0679	0·0340
Total	0·0659	0·2460	0·1801

Small Intestine.

(1) A. 100 c.c. milky extract of dog's intestine + 400 c.c. water. B. 100 c.c. same extract + 400 c.c. water containing 5 gm. sodium glycerophosphate.

P_2O_5 in gm.

A	B	Difference
At commencement :		
0·0042	0·0444	0·0402
After 1 day :		
0·0062	0·1506	0·1444
After 2 days :		
0·0061	0·1539	0·1478
After 6 days :		
0·0061	0·1539	0·1478
Total :		
0·0080	0·1864	0·1784

(2) 80 c.c. clear acid extract of cat's intestine + 5 gm. sodium glycerophosphate in water made up to 500 c.c. with water.

P_2O_5 in gm.

At commencement	0·0256
After 17 hours ...	0·0313
,, 24 ,, ...	0·0346
,, 5 days ...	0·0680
,, 12 ,, ...	0·0963
Total	0·1458

(3) 80 c.c. clear acid extract of intestines of 2 cats neutralised with soda + 2·5 gm. sodium glycerophosphate in water made up to 250 c.c. with water.

P_2O_5 in gm.

At commencement	0·0236
After 1 day ...	0·0877
,, 4 days ...	0·1176
,, 9 ,, ...	0·1270
Total	0·1496

Kidney.

(1) A. 100 c.c. clear acid extract of dog's kidneys neutralised with soda + 150 c.c. water. B. 100 c.c. same neutralised extract + 150 c.c. water containing 2·5 gm. sodium glycerophosphate.

P_2O_5 in gm.

A	B	Difference
At commencement :		
0·0080	0·0090	0·0010
After 3 days :		
0·0075	0·0763	0·0688
After 10 days :		
0·0073	0·0838	0·0765
Total :		
0·0076	0·1661	0·1585

(2) A. 200 c.c. extract of sheep's kidneys + 50 c.c. water. B. 200 c.c. same extract + 50 c.c. water containing 2·5 gm. sodium glycerophosphate.

P_2O_5 in gm.

A	B
At commencement :	
0·0256	0·1300
After 1 day :	
0·0321	0·2085
After 4 days :	
0·0332	0·2092
Total :	
0·0406	0·2130

(3) A. 200 c.c. extract of dog's kidneys + 50 c.c. water. B. 200 c.c. same extract + 50 c.c. water containing 2·5 gm. sodium glycerophosphate.

P_2O_5 in gm.

A	B
At commencement :	
0·0071	0·0176
After 1 day :	
0·0084	0·1204
After 5 days :	
0·0092	0·1723
Total :	
0·0241	0·1750

Lung.

A. 200 c.c. extract of dog's lungs + 50 c.c. water. B. 200 c.c. extract of dog's lungs + 50 c.c. water containing 2·5 gm. sodium glycerophosphate.

P_2O_5 in gm.

	A	B	Difference
At commencement	0·0069	0·0148	0·0079
After 1 day ...	0·0110	0·0344	0·0234
,, 2 days ...	0·0128	0·0472	0·0344
,, 4 ,, ...	0·0161	0·0730	0·0569
Total	0·0241	0·2282	0·2041

Castor oil seeds.

(a) 5 gm. ground seeds suspended in 250 c.c. $\frac{N}{10}$ acetic acid containing 2·5 gm. sodium glycerophosphate.

P₂O₅ in gm.

At commencement	0·0089
After 1 day ...	0·1199
,, 2 days ...	0·1395
,, 3 ,, ...	0·1538
,, 8 ,, ...	0·1852

Total 0·2308

(b) 60 c.c. aqueous extract of ground seeds + 200 c.c. water containing 2·5 gm. sodium glycerophosphate.

P₂O₅ in gm.

At commencement	0·0121
After 12 days ...	0·0105

Total ——

(c) 130 c.c. $\frac{N}{10}$ acetic acid extract + 120 c.c. water containing 2·5 gm. sodium glycerophosphate.

P₂O₅ in gm.

At commencement	0·0217
After 1 day ...	0·0888
,, 2 days ...	0·1027
,, 3 ,, ...	0·1109
,, 6 ,, ...	0·1204

Total 0·2143

(d) 100 c.c. $\frac{N}{10}$ acetic acid extract of ground seeds + 150 c.c. water containing 2·5 gm. sodium glycerophosphate.

P₂O₅ in gm.

At commencement	0·0356
After 1 day ...	0·0920
,, 2 days ...	0·1008
,, 7 ,, ...	0·1179
,, 12 ,, ...	0·1263

Total 0·2003

50 c.c. extract contained—

At commencement	0·0477 gm. inorg. P₂O₅
After 12 days ...	0·0504 ,,	,,
Total P₂O₅ ...	0·0520 gm.

Increases are thus due entirely to hydrolysis of the glycerophosphate.

(e) 200 c.c. $\frac{N}{10}$ acetic acid extract of ground seeds freed from fat by extraction with ether and kept for several months so as to convert all organic phosphorus into inorganic phosphate + 50 c.c. water containing 2·5 gm. sodium glycerophosphate.

P₂O₅ in gm.

At commencement	0·0410
After 1 day ...	0·0579
,, 2 days ...	0·0711
,, 3 ,, ...	0·0806
,, 6 ,, ...	0·1030

Total   0·2003

(f) Residue of seeds in expt. (c) thoroughly washed by decantation and filtration suspended in 250 c.c. water containing 2·5 gm. sodium glycerophosphate.

P₂O₅ in gm.

At commencement	0·0165
After 3 days ...	0·0651
,, 10 ,, ...	0·0889
,, 20 ,, ...	0·1011

Total 0·1826

(g) Residue of seeds in expt. (e) after thorough washing was suspended in 250 c.c. of 1 per cent. solution of sodium glycerophosphate in water.

P₂O₅ in gm.

At commencement	0·0005
After 2 days ...	0·0259
,, 4 ,, ...	0·0338
,, 7 ,, ...	0·0522

Total 0·1500

Yeast.

(*a*) 80 c.c. extract of yeast + 170 c.c. water containing 2·5 gm. sodium glycerophosphate.

P$_2$O$_5$ in gm.

At commencement	0·0396
After 1 day ...	0·0776
,, 3 days ...	0·0869
,, 7 ,, ...	0·0952

Total 0·2244

50 c.c. yeast extract contained—

0·0804 gm. inorg. P$_2$O$_5$
0·0930 gm. total P$_2$O$_5$

Increases are therefore due to hydrolysis of glycerophosphate.

(*b*) A. 10 gm. zymin suspended in 250 c.c. water. B. 10 gm. zymin suspended in 250 c.c. water containing 2·5 gm. sodium glycerophosphate.

P$_2$O$_5$ in gm.

	A	B	Difference
At commencement ...	0·0215	0·0847	0·0632
After 1 day ...	0·0527	0·1323	0·0796
,, 2 days	0·0582	0·1424	0·0842
,, 6 ,,	0·0632	0·1539	0·0907
Total	0·0850	0·2637	0·1787

(*c*) A. 100 c.c. extract of zymin + 150 c.c. water. B. 100 c.c. extract of zymin + 150 c.c. water containing 2·5 gm. sodium glycerophosphate.

P$_2$O$_5$ in gm.

	A	B	Difference
At commencement	0·0304	0·0774	0·0470
After 1 day ...	0·0341	0·0907	0·0566
,, 8 days ...	0·0379	0·1068	0·0689
Total	0·0621	0·2409	0·1788

Bran.

(1) 200 c.c. extract of bran + 50 c.c. water containing 2·5 gm. sodium glycerophosphate.

P$_2$O$_5$ in gm.

At commencement	0·1430
After 1 day ...	0·1696
,, 2 days ...	0·1839
,, 4 ,, ...	0·1877

Total 0·2904

(2) A. 125 c.c. extract of bran + 125 c.c. water. B. 125 c.c. extract of bran + 125 c.c. water containing 2·5 gm. sodium glycerophosphate.

P$_2$O$_5$ in gm.

	A	B	Difference
At commencement ...	0·0374	0·0389	0·0015
After 2 days	0·0389	0·0731	0·0342
,, 4 ,,	0·0389	0·0852	0·0463
,, 9 ,,	0·0393	0·1014	0·0621
Total	0·0406	0·1965	0·1559

The gradual increase in the amount of inorganic P$_2$O$_5$, which occurred when the extracts of intestinal mucosa, kidney, castor oil seeds, yeast and bran were tested, is the best evidence that the hydrolysis of glycerophosphoric acid has been effected by an enzyme present in the tissues. The enzyme is not present in the pancreas and liver of animals. Though an increase in the amount of inorganic phosphate was found in the experiment with pancreatic juice activated by 4–5 drops of intestinal juice, the increase takes place very slowly; one-fifth is hydrolysed in 2 to 3 days, and after 36 days the hydrolysis is less than one-half of the total P$_2$O$_5$ in the solution. An explanation of

this increase is given by the experiments with intestinal extract, which contains the active enzyme; a small amount of the enzyme had therefore been added when activating the pancreatic juice. The absence of the enzyme from the extracts of pancreas and liver, which were prepared specially for lipase, and the presence of the enzyme in the extracts of the intestine and kidney, tissues not usually regarded as good sources of lipase, point to this enzyme being distinct from lipase. Proof of the difference of the enzymes is given by the experiment with castor oil seeds; lipase is insoluble in water but an aqueous extract contains glycerophosphatase. Its presence in bran, in which lipase has not yet been shown, is further proof that the two enzymes are not identical. A comparison of the actual amounts of the enzymes present in the several tissues is not possible, but it may be noticed that the amount of glycerophosphatase in the lung is very much less than in the intestine and kidney. The amount in yeast is also small and the amount contained in bran is smaller still.

II. Phytic acid.

Phytin, the calcium magnesium salt of inositolphosphoric acid, or phytic acid, was first isolated by Posternak in 1903 from various seeds in which it is present as a reserve material containing phosphorus in organic combination. It also occurs in the bran of cereals [Hart and Andrews, 1903], that of rice being particularly rich in it [Suzuki and Yoshimura, 1907]. Phytin is thus the most important phosphorus-containing foodstuff for herbivora, and it has been administered to man to supply organically combined phosphorus. On this account more work has been done upon its metabolism than upon that of any of the other organic phosphorus compounds.

It has been shown by Scofone [1905], Giascosa [1905], Mendel and Underhill [1906], and also Horner [1907] that phytin, when given to animals by the mouth, is absorbed and that the phosphorus is excreted as inorganic phosphorus in the urine; but the absorption is never complete.

Suzuki and Takaishi [1907] have found that bran contains an enzyme, phytase, which completely hydrolyses phytin into inositol and phosphoric acid; Jordan, Hart and Patten [1906] have shown that the enzymes, pepsin and trypsin, of the animal body have no action on phytin, but according to McCollum and Hart [1908], the liver and also the blood contain a phytin-splitting enzyme.

The hydrolysis of glycerophosphoric acid by the enzymes of the intestinal mucosa suggested that phytic acid might also be split up by this tissue. Experiment, however, showed the contrary. Neither the mucous membrane

of the intestine of the carnivora, dog and cat, nor that of the herbivora, sheep, ox and rabbit, even when the latter animal was fed for some weeks on bran, contained an enzyme which was able to hydrolyse phytic acid. Phytic acid thus behaved very differently from glycerophosphoric acid. The difference in the behaviour of the two compounds does not extend to the enzymes of the liver. The hydrolysis of phytic acid by the liver observed by McCollum and Hart was very small and it has been impossible to regard this as a hydrolysis when compared with other results. Phytic acid is thus not hydrolysed by the enzymes of the digestive tract of animals.

The enzymes in an extract of zymin have no action upon phytin, but it is slowly hydrolysed by an acid extract of castor oil seeds. The only enzyme which readily hydrolyses phytin is contained in an extract of bran.

<div align="center">EXPERIMENTAL.</div>

Commercial phytin was employed as the source of phytic acid in these experiments. This substance except for a small residue is easily soluble in water, to which it gives a distinctly acid reaction to litmus, and from which it is precipitated on rendering the solution alkaline with ammonia. It contains a small quantity of inorganic phosphate. The calcium was precipitated from the aqueous solution of phytin by adding the calculated quantity of oxalic acid, or of sodium or potassium oxalate. The solution of the free acid, or of the sodium or potassium salt, obtained on filtering was used as substrate in the experiments.

Extracts of the various tissues were prepared as described under glycerophosphoric acid; the mixture of enzyme solution and phytate was kept at 37° C. in the presence of toluene. Samples were removed at intervals and the inorganic P_2O_5 estimated.

In the first experiments the estimation of inorganic phosphate was carried out by precipitation with ammonium magnesium citrate in the presence of ammonia, but it was soon observed that the inorganic phosphate in the presence of phytic acid was not immediately precipitated but only came down slowly after standing for some time, and that the filtrate frequently gave a further precipitate on standing. The precipitate did not have the usual appearance of ammonium magnesium phosphate and it adhered very tenaciously to the sides of the vessel in which the precipitation took place. On converting these precipitates by heating into the pyrophosphate the weights obtained were also very variable. An examination into the cause of these difficulties revealed the fact that phytic acid inhibits the precipitation

of inorganic phosphate as ammonium magnesium phosphate and that under certain conditions phytic acid also separates as magnesium phytate from ammoniacal solution.

Hart and Andrews [1903], in their study of the relative amounts of inorganic and organic phosphate in foodstuffs, precipitated the inorganic phosphate as ammonium phosphomolybdate at 65° C. in the presence of dilute nitric acid. This method was therefore used but the precipitation was carried out at room temperature since it was thought that the phytic acid might be hydrolysed by the nitric acid at 65° C. A slight hydrolysis does occur at this temperature (see the following paper), but it is insufficient to vitiate the results of Hart and Andrews, or those of McCollum and Hart, who employed this method. The precipitation of the ammonium phosphomolybdate at the ordinary room temperature takes place slowly, but is generally complete in 1–2 days. The completion of the precipitation is shown by the colour of the solution; it is yellow when the reagents are added and becomes quite colourless when the deposition of the yellow precipitate is finished. In the presence of organic matter the solution may remain yellow, which is due to the action of nitric acid on protein in the enzyme extracts. At the same time protein matter is precipitated so that the estimation cannot be carried out by titration as in Neumann's method. When the precipitation was complete the precipitate was filtered off and washed with water and then dissolved in dilute caustic soda. Inorganic phosphate was then precipitated by ammonium magnesium citrate. The details of these experiments will be described in a later paper dealing with the analysis of phytin. Total P_2O_5 was estimated by Neumann's method.

Pancreas.

130 c.c. pancreatic juice + 130 c.c. sodium phytate solution.

P_2O_5 in gm.

At commencement	0·0025
After 14 days ...	0·0055
Total	0·0850

Liver.

A. 125 c.c. extract of dog's liver + 125 c.c. H_2O. B. 125 c.c. extract of dog's liver + 125 c.c. sodium phytate solution.

P_2O_5 in gm.

	A	B	Difference
At commencement ...	0·0348	0·0408	0·0060
After 1 day ...	0·0351	0·0454	0·0103
„ 5 days...	0·0379	0·0486	0·0107
„ 12 „ ...	0·0376	0·0516	0·0140
Total	0·0469	0·1407	0·0938

Intestine.

(1) 50 c.c. extract of cat's intestine (which hydrolysed trypsin digest of caseinogen) + 200 c.c. phytin solution.

P$_2$O$_5$ in gm.

At commencement		0·0126
After 1 day	...	0·0145
,, 2 days	...	0·0158
,, 4 ,,	...	0·0180
Total 0·1154		

(2) 100 c.c. extract of dog's intestine + 150 c.c. phytin solution.

P$_2$O$_5$ in gm.

At commencement		0·0190
After 5 days	...	0·0179
Total 0·1116		

(3) 150 c.c. extract of dog's intestine + 100 c.c. sodium phytate solution.

P$_2$O$_5$ in gm.

At commencement		0·0256
After 1 day	...	0·0264
,, 2 days	...	0·0283
,, 9 ,,	...	0·0306
Total 0·1154		

(4) 200 c.c. extract of ox intestine + 100 c.c. sodium phytate solution.

P$_2$O$_5$ in gm.

At commencement		0·0198
After 1 day	...	0·0219
,, 3 days	...	0·0251
,, 5 ,,	...	0·0264
,, 8 ,,	...	0·0277
Total 0·0964		

(5) 150 c.c. extract of sheep's intestine + 120 c.c. sodium phytate solution.

P$_2$O$_5$ in gm.

At commencement		0·0137
After 6 days	...	0·0139
Total 0·0913		

(6) 100 c.c. extract of rabbit's intestine + 150 c.c. sodium phytate solution.

P$_2$O$_5$ in gm.

At commencement		0·0085
After 6 days	...	0·0106
Total 0·0964		

(7) 150 c.c. extract of intestine of rabbit fed for 3 weeks on bran + 100 c.c. sodium phytate solution.

P$_2$O$_5$ in gm.

At commencement		0·0188
After 2 days	...	0·0267
,, 5 ,,	...	0·0299
,, 8 ,,	...	0·0301
Total 0·1065		

Castor oil seeds.

(a) 150 c.c. acetic acid extract + 100 c.c. sodium phytate solution.

P$_2$O$_5$ in gm.

At commencement		0·0134
After 1 day	...	0·0190
,, 2 days	...	0·0230
,, 4 ,,	...	0·0291
Total 0·1103		

(b) 100 c.c. acetic acid extract + 150 c.c. sodium phytate solution.

P$_2$O$_5$ in gm.

At commencement		0·0188
After 1 day	...	0·0279
,, 4 days	...	0·0458
,, 8 ,,	...	0·0554
,, 12 ,,	...	0·0633
Total 0·1458		

Yeast.

(1) A. 100 c.c. extract of zymin + 150 c.c. water. B. 100 c.c. extract of zymin + 150 c.c. sodium phytate solution.

P$_2$O$_5$ in gm.

	A	B	Difference
At commencement ...	0·0291	0·0362	0·0071
After 7 days...	0·0362	0·0412	0·0050
Total	0·0583	0·1509	0·0926

(2) A. 100 c.c. extract of zymin + 150 c.c. water. B. 100 c.c. extract of zymin + 150 c.c. sodium phytate solution.

P$_2$O$_5$ in gm.

	A	B	Difference
At commencement ...	0·0330	0·0373	0·0043
After 9 days...	0·0359	0·0411	0·0052
Total	0·0579	0·1420	0·0841

Bran.

A. 125 c.c. extract + 125 c.c. water.   B. 125 c.c. extract + 125 c.c. sodium phytate solution.

P_2O_5 in gm.

	A	B	Difference
At commencement	0·0513	0·0566	0·0053
After 1 day ...	0·0531	0·1087	0·0556
„ 4 days ...	0·0559	0·1354	0·0795
„ 10 „ ...	0·0559	0·1354	0·0795
Total	0·0609	0·1510	0·0901

These figures show such a marked difference in the extent of hydrolysis by the extract of bran and by the other enzymes that it can scarcely be concluded that phytase exists in any of the extracts except in that of castor oil seeds; the hydrolysis in this case is very slow; only a very slight increase in the amount of inorganic phosphate is noticeable with the intestinal and liver extracts, and none with pancreatic juice and extract of zymin.

III.   Hexosephosphoric acid.

Harden and Young [1908] have found that hexosephosphoric acid is formed during the fermentation of glucose and fructose by yeast or by zymase, and that it is subsequently hydrolysed. They attribute the hydrolysis to a special enzyme, hexosephosphatase. Harding [1912] has recently investigated the action of enzymes upon hexosephosphate and has observed that an enzyme present in castor oil seeds hydrolyses it, and that this enzyme is not present in ox pancreas. These data are so closely parallel to those obtained with glycerophosphoric acid that it was very important to test the action of the intestine and of bran upon this compound. Extracts of both these tissues were found to contain hexosephosphatase. The parallelism between the action of glycerophosphatase and hexosephosphatase is thus extremely close, and it would seem that the same phosphatase hydrolyses both these compounds.

Experimental.

The hexosephosphoric acid employed in these two experiments was most kindly supplied to me by Dr Young. The solution of hexosephosphoric acid was neutralised and mixed with a neutral extract of intestinal mucous membrane and extract of bran, both of which were prepared by the methods described under glycerophosphoric acid. Toluene was added and the mixture kept at 37° C. The inorganic P_2O_5 was estimated in samples by precipitation

with ammonium magnesium citrate and converted into magnesium pyro-phosphate.   Total P_2O_5 in the solution was estimated by Neumann's method with another sample.

(a) *Intestinal Extract.*

100 c.c. extract + 160 c.c. hexosephosphate solution.

P_2O_5 in gm.

At commencement	...	...	0·0435
After 2 days ...	...	...	0·0815
,, 4 ,, ...	...	...	0·0819

Total 0·0860

P_2O_5 in intestinal extract added per 50 c.c. sample = 0·0146 gm.

(b) *Bran Extract.*

A.   100 c.c. bran extract + 150 c.c. water.   B.   100 c.c. hexosephosphate solution + 150 c.c. water.   C.   100 c.c. bran extract + 100 c.c. hexosephosphate solution + 50 c.c. water.

	A Bran extract alone	B Hexosephosphate alone	C Hexosephosphate + bran extract	Difference between C and A + B
At commencement	0·0225	0·0092	0·0352	0·0035
After 1 day ...	0·0236	0·0093	0·1006	0·0677
,, 2 days ...	0·0240	0·0110	0·1115	0·0765
,, 4 ,, ...	0·0244	0·0121	0·1188	0·0823
Total	0·0279	0·1801	0·2054	—

Hydrolysis has occurred with both intestinal extract and bran extract; it is most marked in the case of intestinal extract where it was complete in two days.   Bran extract caused a marked hydrolysis in one day but it was not complete in four days, in which time scarcely half of the compound was hydrolysed.

IV.   ETHYL PHOSPHORIC ACID AND DIETHYL PHOSPHORIC ACID.

The action of enzymes upon the simple esters of phosphoric acid has never been investigated.   Ethyl phosphoric acid and diethyl phosphoric acid were therefore tested against those extracts of tissues which had been found to hydrolyse glycerophosphoric acid, phytic acid and hexosephosphoric acid. The parallelism in the action of the enzyme upon glycerophosphoric and hexosephosphoric acid occurred again in their action upon ethyl phosphoric acid; diethyl phosphoric acid was not hydrolysed by intestinal extract nor by extract of castor oil seeds; unfortunately the quantity of diethyl phosphoric acid available was not sufficient to test against the other extracts.

The material used in these investigations was prepared either by the

action of phosphoric pentoxide or of phosphorus oxychloride upon absolute alcohol. The product of the reaction in each case was neutralised with excess of baryta; barium phosphate was filtered off and the clear filtrate precipitated by an equal volume of alcohol; barium ethyl phosphate was precipitated in crystalline form and recrystallised from water and alcohol. This was converted into the sodium salt by treatment with the calculated quantity of sodium sulphate and the solution so obtained was used in the experiments with the enzymes. A small quantity of barium diethyl phosphate was contained in the alcoholic filtrate obtained in the preparation by means of phosphorus pentoxide; it crystallised out on concentration of the alcoholic solution and was purified by recrystallisation from dilute alcohol. The solution of the sodium salt prepared by treatment of the barium salt with sodium sulphate was employed for the investigation of the action of the enzymes.

EXPERIMENTAL.

The enzyme solutions were prepared in the same way as described under glycerophosphoric acid. Inorganic phosphate was estimated by precipitation with ammonium magnesium citrate and conversion into magnesium pyrophosphate. The experiments were the following:—

A. *Sodium Ethyl Phosphate.*

Pancreas.

0·5 gm. trypsin (Fairchild) was dissolved in 110 c.c. deci-normal sodium carbonate solution and filtered. 100 c.c. extract + 200 c.c. sodium ethyl phosphate solution.

P_2O_5 in gm.

At commencement	0·0052
After 11 days ...	0·0046
Total 0·0659	

Intestine.

(1) 125 c.c. neutral intestinal extract + 130 c.c. sodium ethyl phosphate solution.

P_2O_5 in gm.

At commencement	0·0168
After 1 day ...	0·0492
,, 2 days ...	0·0572
,, 4 ,, ...	0·0628
Total 0·0654	

(2) 110 c.c. neutral intestinal extract + 150 c.c. sodium ethyl phosphate solution.

P_2O_5 in gm.

At commencement	0·0121
After 1 day ...	0·0457
,, 2 days ...	0·0560
,, 4 ,, ...	0·0626
Total 0·0672	

Castor oil seeds.

(1) 125 c.c. acetic acid extract of seeds + 130 c.c. sodium ethyl phosphate solution.

P_2O_5 in gm.

At commencement	0·0070
After 1 day ...	0·0235
,, 2 days ...	0·0307
,, 4 ,, ...	0·0383
Total 0·0697	

(2) 110 c.c. neutralised extract of castor oil seeds + 150 c.c. sodium ethyl phosphate solution.

P_2O_5 in gm.

At commencement	0·0075
After 1 day ...	0·0135
,, 4 days ...	0·0177
Total 0·0735	

Yeast.

60 c.c. extract of zymin + 200 c.c. sodium ethyl phosphate solution.

P₂O₅ in gm.	
At commencement	0·0111
After 1 day ...	0·0283
,, 5 days ...	0·0398
,, 12 ,, ...	0·0491
Total 0·1052	

Bran.

60 c.c. extract of bran + 200 c.c. sodium ethyl phosphate solution.

P₂O₅ in gm.	
At commencement	0·0148
After 1 day ...	0·0190
,, 2 days ...	0·0216
,, 5 ,, ...	0·0259
,, 12 ,, ...	0·0319
Total 0·0938	

B. *Sodium Diethyl Phosphate.*

Intestine.

150 c.c. acid extract of intestinal mucosa + 100 c.c. sodium diethyl phosphate solution: after 3 days solution neutralised with a few drops of sodium carbonate solution.

P₂O₅ in gm.	
At commencement	0·0250
After 13 days ...	0·0250
Total 0·0888	

Castor oil seeds.

150 c.c. neutralised acid extract + 100 c.c. sodium diethyl phosphate solution: after 4 days acidified with 2 drops of glacial acetic acid.

P₂O₅ in gm.	
At commencement	0·0138
After 11 days ...	0·0156
Total 0·0875	

Sodium ethyl phosphate is hydrolysed most readily by the extract of the intestinal mucosa and it is hydrolysed by an acid extract of castor oil seeds; the hydrolysis also takes place where the extract is neutralised.

Extracts of yeast and of bran also contain an enzyme which can hydrolyse it; in the case of bran the hydrolysis is very slow.

Sodium diethyl phosphate was not hydrolysed by either intestinal extract or extract of castor oil seeds.

V. Hydroxymethyl-phosphinic acid ($OH \cdot CH_2 \cdot PO_3 \cdot H$).

The previous results of the action of enzymes upon compounds which are esters of phosphoric acid led to an investigation of their action upon hydroxy-methyl-phosphinic acid, a compound in which the phosphoric acid is linked to carbon instead of through oxygen as in the other compounds.

This compound was prepared by Mr H. J. Page [1912] in this laboratory and a quantity was kindly placed at my disposal for this purpose. No natural organic phosphorus compounds of this constitution are known; these experiments were consequently of further interest, since if hydroxymethyl-phosphinic acid were hydrolysed by enzymes, natural organic compounds of this constitution might be expected to occur in nature.

EXPERIMENTAL.

The solution of the free acid was exactly neutralised and mixed with the enzyme solution which was prepared as previously described; the analysis was carried out in the same way as with glycerophosphoric acid.

The results were:

(a) Action of Trypsin.

105 c.c. $\frac{N}{10}$ sodium carbonate extract of 0·5 gm. trypsin (Fairchild) + 150 c.c. sodium hy-droxymethyl-phosphinate solution.

P₂O₅ in gm.

At commencement	0·0046
After 7 days ...	0·0055
Total 0·1344	

(b) Action of Extract of Intestinal Mucosa.

100 c.c. neutralised extract of dog's intesti-nal mucosa + 150 c.c. sodium hydroxymethyl-phosphinate solution.

P₂O₅ in gm.

At commencement	0·0131
After 4 days ...	0·0144
Total 0·2422	

(c) Action of Extract of Castor oil seeds.

100 c.c. acetic acid extract of castor oil seeds + 150 c.c. sodium hydroxymethyl-phos-phinate solution.

P₂O₅ in gm.

At commencement	0·0009
After 4 days ...	0·0019
Total 0·2840	

(d) Action of Extract of Bran.

100 c.c. extract of bran + 150 c.c. sodium hydroxymethyl-phosphinate solution.

P₂O₅ in gm.

At commencement	0·0508
After 4 days ...	0·0526
Total 0·2244	

(e) Action of Extract of Zymin.

(1) 100 c.c. extract of zymin + 150 c.c. sodium hydroxymethyl-phosphinate solution.

P₂O₅ in gm.

At commencement	0·0393
After 1 day ...	0·0487
,, 2 days ...	0·0509
,, 4 ,, ...	0·0528
,, 7 ,, ...	0·0549
Total 0·2333	

(2) A. 100 c.c. extract of zymin + 150 c.c. H₂O as control. B. 100 c.c. extract of zymin + 150 c.c. sodium hydroxymethyl-phosphinate solution.

P₂O₅ in gm.

	A	B	Difference
At commence-ment ...	0·0216	0·0227	0·0011
After 2 days...	0·0296	0·0362	0·0066
,, 5 ,, ...	0·0310	0·0393	0·0083
,, 8 ,, ...	0·0329	0·0424	0·0095
Total	0·0583	0·1977	0·1394

As was expected this compound was not hydrolysed by any of the extracts which contain glycerophosphatase even if we include the action of the extract of zymin; in this case an increase of inorganic P₂O₅ was observed at the commencement but the increase was very slight later on; it is probably due to a more rapid separation of P₂O₅ in the enzyme solution arising from a change in reaction when the mixture was made.

VI. Nucleic acid.

The action of the enzymes of animal organs upon various nucleic acids has been studied by many investigators, but their attention has been confined almost entirely to the separation of the purine bases from the complex molecule, and little or no attention has been given to the separation of the phosphoric acid.   Milroy [1896] found that 10–16 per cent. of the total phosphorus in a peptic digest of nuclein was inorganic phosphoric acid, and that in a trypsin digest the amount of inorganic phosphoric acid varied from 6–47 per cent. of the total phosphorus content.   No estimation of the amount of the phosphoric acid present in the enzyme solution was made, so that a portion or the whole of the inorganic phosphate might have been so introduced into his solutions.   Levene and Medigreceanu [1911] in some of their recent papers state that the enzymes of the intestinal mucosa hydrolyse nucleic acid with the liberation of phosphoric acid; no estimation of the amount of the inorganic phosphate was made.   The formation of phosphoric acid during the autolysis of yeast, first observed by Béchamps in 1865, was attributed to the hydrolysis of nucleic acid; hexosephosphate and hexose-phosphatase were unknown at that time and it is most probable that the phosphoric acid observed by Béchamps originated from this compound rather than from nucleic acid.

There is thus no satisfactory proof that inorganic phosphate is liberated by enzymes from nucleic acid though it is very probable.   The following experiments were therefore carried out to test these earlier observations and to compare the hydrolysis of nucleic acid by the same enzymes which have been found to liberate phosphoric acid from the other organic phosphorus compounds.

It has not been possible to test all the various nucleic acids but the results show that an extract of intestinal mucosa slowly separates inorganic phosphoric acid from thymus nucleic acid and from wheat nuclein, but more rapidly and completely from the organic phosphorus compound (inosinic acid) in meat extract; trypsin does not hydrolyse thymus nucleic acid, nor the compound in meat extract.   An extract of zymin showed no appreciable separation of phosphoric acid from the thymus nucleic acid, whereas an extract of bran hydrolysed it.

Experimental.

The thymus nucleic acid used in these experiments was prepared for me by Miss R. F. Skelton by Kossel's method [1894].   The preparation was almost pure white in colour and contained 8 per cent. of phosphorus and

17 per cent. of nitrogen. It dissolved readily in decinormal sodium carbonate giving a solution which was faintly brown in colour.

The meat extract was a solution of lemco, and the wheat nuclein was also kindly prepared for me by Miss Skelton from wheat germ by digestion with pepsin hydrochloric acid. The insoluble residue of nuclein was dissolved in dilute sodium carbonate, separated from starch by centrifugalisation and then reprecipitated by acid. After washing with water it was dissolved in decinormal sodium carbonate solution and the brown solution thus obtained was employed.

The enzyme extracts were prepared in the same way as those used in investigating the hydrolysis of glycerophosphoric acid; the extract was mixed with the solution of substrate and kept in the presence of toluene at 37° C. Samples were removed and inorganic P_2O_5 estimated by precipitation with ammonium magnesium citrate. Total P_2O_5 was estimated in another sample by Neumann's method.

(a) Action of Extract of Pancreas.

(1) 2 gm. trypsin (Fairchild) were dissolved in 260 c.c. $\frac{N}{10}$ sodium carbonate solution and filtered. A. 125 c.c. extract + 125 c.c. water. B. 125 c.c. extract + 125 c.c. solution containing 2·3 gm. thymus nucleic acid dissolved in $\frac{N}{10}$ sodium carbonate.

P_2O_5 in gm.

	A	B
At commencement	0·0071	0·0070
After 1 day ...	0·0074	0·0087
,, 11 days ...	0·0074	0·0089
Total	0·0139	0·0951

(2) 0·5 gm. trypsin (Fairchild) dissolved in 110 c.c. $\frac{N}{10}$ sodium carbonate solution and filtered. 100 c.c. extract + 150 c.c. of a solution containing 10 gm. of lemco.

P_2O_5 in gm.

At commencement	0·1189
After 7 days ...	0·1194
Total	0·1331

(b) Action of Extract of Intestine.

(1) 100 c.c. neutralised extract of intestinal mucous membrane of dog + 150 c.c. solution containing 2 gm. thymus nucleic acid in $\frac{N}{10}$ sodium carbonate.

P_2O_5 in gm.

At commencement	0·0152*
After 1 day ...	0·0254
,, 2 days ...	0·0277
,, 4 ,, ...	0·0310
,, 7 ,, ...	0·0327
Total	0·0938

* 0·0117 gm. P_2O_5 was contained in the intestinal extract for each sample.

(2) 100 c.c. neutralised extract of intestinal mucous membrane of dog + 200 c.c. solution of wheat nuclein in sodium carbonate neutralised with acetic acid.

P_2O_5 in gm.

At commencement	0·0147
After 1 day ...	0·0211
,, 2 days ...	0·0200
,, 4 ,, ...	0·0228
Total	0·0596

(3) 60 c.c. neutralised extract of intestinal mucous membrane of a dog + 11 gm. lemco dissolved in 200 c.c. water.

P₂O₅ in gm.

At commencement	0·1254
After 1 day ...	0·1458
,, 4 days ...	0·1470

Total 0·1521

(4) 100 c.c. neutralised extract of intestinal mucous membrane of cat + 10 gm. lemco dissolved in 160 c.c. water.

P₂O₅ in gm.

At commencement	0·1409
After 1 day ...	0·1668
,, 5 days ...	0·1712

Total 0·1839

(c) Action of Extract of Zymin.

A. 100 c.c. extract of zymin + 150 c.c. H_2O.
B. 100 c.c. extract of zymin + 150 c.c. of a solution containing 2 gm. thymus nucleic acid in $\frac{N}{10}$ sodium carbonate.

P₂O₅ in gm.

	A	B	Difference
At commencement ...	0·0216	0·0235	0·0019
After 2 days...	0·0296	0·0322	0·0026
,, 5 ,, ...	0·0310	0·0353	0·0043
,, 8 ,, ...	0·0329	0·0380	0·0051
Total	0·0583	0·1281	0·0698

(d) Action of Extract of Bran.

100 c.c. extract of bran + 150 c.c. solution containing 2 gm. thymus nucleic acid in decinormal sodium carbonate.

P₂O₅ in gm.

At commencement	0·0297*
After 1 day ...	0·0432
,, 2 days ...	0·0498
,, 5 ,, ...	0·0625

Total 0·1091

* 0·0279 gm. was contained in the bran extract for each sample.

Trypsin has no action upon nucleic acid; the action of intestinal extract upon thymus nucleic acid is very slight and is still less upon wheat nuclein; it readily hydrolyses the organic phosphorus compound contained in lemco. Extract of zymin has also a very slight hydrolysing action, but extract of bran is more active. No marked hydrolysis of nucleic acid is noticeable by any of the extracts.

VII. Phosphoprotein.

The separation of the phosphorus of caseinogen by the action of enzymes and by alkali was investigated in 1906 by Plimmer and Bayliss [1906], who confirmed the earlier work of Biffi with regard to the action of trypsin. Pepsin slowly converted the phosphorus of caseinogen into a soluble form, but without the formation of phosphoric acid. Trypsin separated 35 per cent. of the phosphorus as phosphoric acid, the remaining 65 per cent. being present as a soluble organic compound. Papain also converted the phosphorus of caseinogen into a soluble form, but no investigation was made as to whether phosphoric acid was produced.

Complete separation of the phosphorus from caseinogen was thus not effected by trypsin. It seemed however very probable that the enzyme of

the intestinal mucosa, most probably erepsin, which according to Cohnheim hydrolyses caseinogen, would complete the separation as phosphoric acid. This was found to be the case. Papain also separates phosphoric acid from caseinogen in both acid and alkaline media but the separation is very slow. The vegetable proteoclastic enzyme "digestin" prepared by Yenjo in Japan from an *Aspergillus* behaves like papain. Neither an extract of zymin nor an extract of bran separates phosphoric acid from the organic phosphorus compound remaining after the action of trypsin upon caseinogen. It might have been expected that the extract of zymin would hydrolyse this compound and its inactivity is probably accounted for by its method of preparation with acetone, which coagulates the protein to which the proteoclastic enzyme is attached, so that the latter is not extracted by water.

EXPERIMENTAL.

A trypsin digest of caseinogen was prepared by digesting 200 gm. pure caseinogen dissolved in 2000 c.c. water containing 10 c.c. ammonia of sp. gr. 0·880 with 3 gm. trypsin (Fairchild) for two months at 37° in the presence of 20 c.c. of toluene. The solution was no longer alkaline to phenolphthalein after one month and ammonia was added till the solution became just red to this indicator. At the end of this time the tyrosine which had separated was filtered off. The clear brown solution contained 0·0456 gm. total P_2O_5 and 0·0209 gm. inorganic P_2O_5 per 50 c.c. 56 per cent. of the total P_2O_5 was thus present as organic P_2O_5. A portion of this solution which was kept for another four months was found to contain 0·0418 gm. total P_2O_5 and 0·0228 gm. inorganic P_2O_5 per 50 c.c. A year later the amounts were 0·0430 gm. total P_2O_5 and 0·0242 gm. inorganic P_2O_5 i.e. the solution contained 45 per cent. organic phosphorus.

Hydrolysis thus proceeds but even after 18 months it is not complete.

The action of the extracts of the intestinal mucosa, bran and zymin was investigated in the same way as described under glycerophosphoric acid. The action of papain and digestin was investigated with caseinogen which was dissolved in dilute caustic soda solution (20 gm. caseinogen + 40 c.c. semi-normal NaOH + 500 c.c. water): one half of this mixture was used for the experiment in alkaline solution and 200 c.c. of the remainder were acidified with 25 c.c. of semi-normal sulphuric acid and employed for the experiment in acid solution. Samples were removed at intervals and filtered for the estimation of the inorganic P_2O_5 and total P_2O_5. The action of papain was also tested upon the above trypsin digest of caseinogen.

The experiments were:

Action of Intestinal Extract.

(a) 125 c.c. extract of cat's intestine + 125 c.c. trypsin digest of caseinogen.

P₂O₅ in gm.	
At commencement	0·0223
After 3 days ...	0·0500
,, 5 ,, ...	0·0492
Total	0·0579

(b) 50 c.c. acid extract of cat's intestine + 200 c.c. trypsin digest of caseinogen.

P₂O₅ in gm.	
At commencement	0·0224
After 18 hours ...	0·0302
,, 24 ,, ...	0·0313
,, 42 ,, ...	0·0325
,, 4 days ...	0·0338
Total	0·0406

(c) 50 c.c. acid extract of dog's intestine + 200 c.c. trypsin digest of caseinogen.

P₂O₅ in gm.	
At commencement	0·0244
After 17 hours ...	0·0329
,, 65 ,, ...	0·0357
,, 6 days ...	0·0367
Total	0·0431

Action of Papain.

(a) In acid solution on caseinogen.

P₂O₅ in gm.	
After 38 days	0·0037
,, 64 ,,	0·0052
Total	0·0203

(b) In alkaline solution on caseinogen.

P₂O₅ in gm.	
After 38 days	0·0075
,, 64 ,,	0·0092
Total	0·0349

(c) On trypsin digest. 200 c.c. trypsin digest + 55 c.c. extract of papain.

P₂O₅ in gm.	
At commencement	0·0166
After 1 day ...	0·0168
,, 2 days ...	0·0166
,, 3 ,, ...	0·0168
Total	0·0368

Action of Digestin (Yenjo).

(a) In acid solution on caseinogen.

P₂O₅ in gm.	
After 4 months	0·0124
Total	0·0203

(b) In alkaline solution.

P₂O₅ in gm.	
After 4 months	0·0236
Total	0·0330

Action of Extract of Zymin.

A. 200 c.c. trypsin digest + 50 c.c. extract.
B. 200 c.c. water + 50 c.c. extract.

P₂O₅ in gm.	A	B	Difference
At commencement ...	0·0286	0·0085	0·0201
After 2 days...	0·0316	0·0131	0·0185
,, 4 ,, ...	0·0282	0·0139	0·0143
Total	0·0634	0·0279	0·0355

Action of Extract of Bran.

A. 200 c.c. trypsin digest + 50 c.c. extract.
B. 200 c.c. water + 50 c.c. extract.

P₂O₅ in gm.	A	B	Difference
At commencement ...	0·0338	0·0142	0·0196
After 2 days...	0·0315	0·0148	0·0167
,, 4 ,, ...	0·0327	0·0147	0·0180
Total	0·0494	0·0190	0·0304

In comparison with the other extracts that of the intestinal mucosa is the most active in separating phosphoric acid from caseinogen. The hydrolysis is almost complete within 24 hours; in all these experiments a small quantity remained in an organic form. This is most probably due to impurity and corresponds to the small quantity of organic phosphorus which always remains

when phosphoprotein is decomposed by alkali, as was found by Plimmer and Scott [1908].

Papain in alkaline solution separated one quarter of the total P_2O_5 in 35 days and this amount was slightly increased in 64 days; in acid solution in the same time one fifth was separated as inorganic phosphate, which amount was increased to one quarter in 64 days; further separation does not seem to occur as papain does not hydrolyse the organic P_2O_5 present in the trypsin digest of caseinogen. Digestin also never separated the whole of the phosphorus of caseinogen. Neither extract of bran nor extract of zymin separated phosphoric acid from the organic phosphorus in a trypsin digest.

SUMMARY AND CONCLUSIONS.

The action of enzymes upon the organic phosphorus compounds is best summarised by a tabulation of the results:

	Pancreas	Liver	Intestine	Castor oil seeds	Yeast (Zymin)	Bran
Glycerophosphoric acid ...	0	0	+	+	+	+
Hexosephosphoric ,, ...	0	...	+	+	+	+
Ethyl phosphoric ,, ...	0	...	+	+	+	+
Diethyl phosphoric ,, ...	...	...	0	0	...	...
Phytic acid ...	0	0	0	+	0	+
Nucleic acid (Thymus) ...	0	...	+	...	+ ?	+
,, ,, (Wheat) ...	...	...	+ ?	. .	. .	. .
,, ,, (Meat) ...	0	...	+	...		
Hydroxymethylphosphinic acid	0	...	0	0	+ ?	0
Phosphoprotein	⅜	...	+	...	0	0

The most active tissue in the hydrolysis of the organic phosphorus compounds is the intestinal mucosa; all the organic phosphoric compounds except phytic acid were hydrolysed by it. Diethyl phosphoric acid and hydroxymethyl-phosphinic acid must be excluded entirely as they were not hydrolysed by any extract. Phytic acid and phosphoprotein stand out in marked contrast with the other compounds. Phytic acid is attacked readily only by the enzyme in bran extract. Phosphoprotein is the only compound which is hydrolysed by the pancreas. The constitution of phosphoprotein is different from that of the other compounds: it is a protein and is hydrolysed by the proteoclastic enzymes and it seems most likely that the separation of the phosphoric acid is effected by the same enzyme which unlinks the amino-acids from one another. The other organic phosphorus compounds examined are esters of phosphoric acid. The inactivity of the animal tissues most rich in lipase and the activity of an extract of castor oil seeds which does not contain lipase shows that the lipoclastic enzymes have no action on these

esters and that they are hydrolysed by a special group of enzymes.   Are we to consider that these esters of phosphoric acid are all hydrolysed by one enzyme, or are we to consider that each compound is hydrolysed by a specific phosphatase?   If there were only one phosphatase then phytic acid should be hydrolysed by the enzyme of the intestine, but as this is not the case we must regard phytase as a specific enzyme.   The varying extent of the hydrolysis of the other compound by the different extracts supports the supposition that there is a specific enzyme for each phosphoric ester.   It can scarcely, however, be admitted that the specificity is really so great.   If the chemical constitution of each of the compounds be considered it is seen that glycerophosphoric acid and ethyl phosphoric acid are simple esters, hexose-phosphoric acid is regarded as hexosediphosphoric acid and nucleic acid is a complex made up of hexose or pentose mono-phosphoric acid.

One enzyme is supposed to hydrolyse the group of α-glucosides, another enzyme the group of β-glucosides, invertase separates fructose from sucrose and raffinose, trypsin attacks the polypeptide linking of the amino-acids; consequently it can scarcely be conceived that the specificity of the phosphatases extends to every ester and it would be better to group the phosphatases into several classes such as mono-phosphatases which attack mono-esters like glycerophosphoric acid and ethyl phosphoric acid, di-phosphatases which attack di-esters like hexosephosphoric acid, hexa-phosphatases which attack hexa-esters like phytic acid.   Glycerophosphatase will hydrolyse both glycerophosphoric acid and ethyl phosphoric acid as well as other simple esters.   Proof of such a supposition will only be given by further work when more simple esters have been examined and when the constitution of hexosephosphoric acid, phytic acid, and nucleic acid have been definitely established.

These experiments confirm the work of Fingerling and Gregersen which shows that the animal organism can synthesise its organic phosphorus compounds from inorganic phosphates.   They are not in favour of the belief that organic phosphorus compounds are essential for the well-being of animals, a belief which has arisen from feeding experiments with inorganic and organic phosphorus.   No proper control was ever made in such experiments.   When phosphoprotein was compared with albumin as the substitute for the supply of protein the nutritive value of albumin was reckoned as equivalent to that of caseinogen, no attention being paid to the different composition of these proteins in amino-acids; when phytic acid, or phytin, was compared with inorganic phosphate the control was not made with inositol and phosphoric acid.   Further, if assimilation of organically combined phosphorus occurred,

both phosphoprotein and phytic acid should be found in the blood and tissues. Phosphoprotein occurs only as the secretion of the mammary gland in mammals and of the yolk gland in birds; in these tissues it is synthesised. Phytin has never been found in animals but inositol has been found in most tissues.

The organic phosphorus compounds except phytic acid are all hydrolysed by the intestinal mucosa and their fate in assimilation is like that of all the foodstuffs :—the proteins are known to be completely broken down into amino-acids before they enter the circulation ; the fats are hydrolysed as they pass through the intestinal wall ; the disaccharides are broken down by enzymes in the mucous membrane of the small intestine. The organic phosphorus compounds are therefore almost certainly assimilated as inorganic phosphate and the organic radicle with which the phosphorus is combined.

The non-hydrolysis of phytic acid by the intestinal mucosa was at first attributed to the experiment being made with carnivora (dog and cat), to which animals vegetable food is not natural, but the same experiment with the herbivora (sheep, ox and rabbit) required some further explanation as to the manner of assimilation of this compound. The excretion of the phosphorus of phytic acid as inorganic phosphate, which was shown by Scofone, Giascosa, Mendel and Underhill and also Horner, pointed to the hydrolysis of the compound. If the absorption be not complete, as the above workers have pointed out, unchanged phytic acid should be present in the faeces. A rabbit was therefore fed for three weeks on food containing a large proportion of bran, and its faeces were examined at the end of this period for organic and inorganic phosphate. The faeces were extracted with dilute hydrochloric acid ; inorganic and total P_2O_5 were estimated in the extract by precipitation with ammonium magnesium citrate and by Neumann's method. The phosphorus present in the extract was almost entirely inorganic P_2O_5, as is shown by the following figures :

| | H_2O | Total P_2O_5 | Extract | | Residue |
			Total P_2O_5	Inorganic P_2O_5	Total P_2O_5
Nov. 30, 1911	14·8 per cent.	1·9 per cent.	1·5 per cent.	1·4 per cent.	0·2 per cent.
Dec. 1	27·7	1·7	1·6	1·4	—
,, 5	30·0	1·7	1·6	1·5	0·2
,, 6	25·6	2·0	2·0	1·9	—
,, 8	35·3	1·9	1·7	1·4	0·2

Hydrolysis of the phytic acid had occurred ; this is not caused by the acid employed in extracting the faeces, for dilute acid at the ordinary temperature does not hydrolyse phytic acid (see following paper). Since the

animal was fed on bran the hydrolysis must be effected by the phytase in the foodstuff. The assimilation of phytic acid by the herbivora thus resembles that of cellulose, which was shown by Horace Brown [1892] to be hydrolysed by the cytase present in the vegetable food.

In carnivora and man, the assimilation of the phosphorus of phytic acid is also in the form of inorganic phosphate. Rogoginski [1910] has shown that in dogs the unabsorbed phytin is present as such in the faeces and that in man the phytic acid is hydrolysed by the bacteria in the large intestine. He also finds no beneficial effect on metabolism from the organic phosphorus compounds. Absorption of phytic acid by animals would require rapid dialysis through the semi-permeable membranes. The dialysis of phytic acid through parchment membranes is slow and is only complete after several days. This is shown by the following experiments:

	26 c.c. sodium phytate inside 50 c.c. water outside P_2O_5 in 5 c.c.		30 c.c. sod. phyt. inside 100 c.c. water outside P_2O_5 in 5 c.c.		70 c.c. sod. phyt. inside 200 c.c. water outside P_2O_5 in 5 c.c.	
	Inside	Outside	Inside	Outside	Inside	Outside
	26·9 mgm.	0 mgm.	31·5 mgm.	0 mgm.	32·5 mgm.	0 mgm.
After 1 day	—	1·5	—	4·4	—	2·7
,, 2 days	—	5·3	—	7·0	—	5·1
,, 3 ,,	—	—	—	7·6	—	6·7
,, 4 ,,	11·5	9·3	8·0	8·0	20·0	6·6

In all probability the membrane of the intestine of animals behaves in the same way as the semi-permeable membrane of plant seeds, which does not allow acids to pass through, as was shown by Adrian Brown [1909].

There is thus no evidence of the absorption of phytic acid; it will be hydrolysed either by the enzyme in the foodstuffs, or by bacteria in the large intestine, and will enter the circulation of animals as inositol and phosphoric acid.

The value of the organic phosphorus compounds in nutrition entirely depends on the nature of the organic matter with which the phosphoric acid is combined.

REFERENCES.

Armstrong and Ormerod (1906), *Proc. Roy. Soc.* 78, B, 376.
Brown, H. T. (1892), *J. Chem. Soc.* 61, 352.
Brown, A. J. (1909), *Proc. Roy. Soc.* 81, B, 82.
Bülow (1894), *Arch. ges. Physiol.* 57, 89.
Cook (1909), *U. S. Department of Agriculture, Bureau of Chemistry, Bulletin*, No. 123.
Connstein, Hoyer and Wartenberg (1902), *Ber.* 35, 3988.
Dietz (1907), *Zeitsch. physiol. Chem.* 52, 279.
Fingerling (1912, 1), *Biochem. Zeitsch.* 38, 448.
Fingerling (1912, 2), *Biochem. Zeitsch.* 39, 239.

Giascosa (1905), *Biochem. Centr.*, **4**, 572.
Gregersen (1907), *Zeitsch. physiol. Chem.* **53**, 453.
Gregersen (1911), *Zeitsch. physiol. Chem.* **71**, 49.
Grosser and Husler (1912), *Biochem. Zeitsch.* **39**, 1.
Harden and Young (1908), *Proc. Roy. Soc.* **80**, B, 299.
Harding (1912), *Proc. Roy. Soc.* **85**, B, 418.
Hart and Andrews (1903), *Amer. Chem. J.* **30**, 470.
Horner (1907), *Biochem. Zeitsch.* **2**, 428.
Jordan, Hart and Patten (1906), *Amer. J. Physiol.* **16**, 268.
Kossel (1894), *Ber.* **27**, 2215.
Levene and Medigreceanu (1911), *J. Biol. Chem.* **9**, 389.
Loevenhart and Peirce (1906), *J. Biol. Chem.* **2**, 397.
Marfori (1905), *Archivio di Fisiol.* **2**, 217.
Mathison (1909), *Biochemical J.* **4**, 274.
McCollum and Hart (1908), *J. Biol. Chem.* **4**, 497.
Mendel and Underhill (1906), *Amer. J. Physiol.* **17**, 75.
Milroy (1896), *Zeitsch. physiol. Chem.* **22**, 307.
Page (1912), *J. Chem. Soc.* **101**, 423.
Power and Tutin (1905), *J. Chem. Soc.* **87**, 249.
Plimmer and Bayliss (1906), *J. Physiol.* **33**, 439.
Plimmer, Dick and Lieb (1909), *J. Physiol.* **39**, 98.
Plimmer and Scott (1908), *J. Chem. Soc.* **93**, 1699.
Posternak (1903), *Compt. rend.* **137**, 202.
Rogozinski (1910), *Chem. Zentr.*, II. 1549.
Scofone (1905), *Biochem. Centr.*, **3**, 606.
Suzuki and Takaishi (1907), *Bull. Coll. Agric. Tokyo*, **7**, 503.
Suzuki and Yoshimura (1907), *Bull. Coll. Agric. Tokyo*, **7**, 495.
Tanaka (1910), *J. Coll. Eng. Tokyo*, **5**, 25.
Tutin and Hann (1906), *J. Chem. Soc.* **89**, 1749.
Willstätter and Lüdecke (1904), *Ber.* **37**, 3753.

V. THE HYDROLYSIS OF ORGANIC PHOSPHORUS COMPOUNDS BY DILUTE ACID AND BY DILUTE ALKALI.

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Plimmer and Scott [1908] have shown that phosphoproteins may be distinguished from other organic phosphorus compounds occurring in animal tissues by their behaviour with dilute caustic soda. The phosphorus of the phosphoproteins is completely separated as inorganic phosphate by one per cent. caustic soda at 37° C. in twenty-four hours. Dilute caustic soda at 37° C. does not decompose the other natural organic phosphorus compounds but their hydrolysis at higher temperatures has never been investigated. Neumann's method of preparation of nucleic acid, in which the tissues are heated with caustic alkali, shows that nucleic acid in comparison with phosphoprotein is much more stable; it is not definitely known if the nucleic acid is hydrolysed by alkali. Hexosephosphoric acid is readily decomposed by alkali. The synthetical esters of phosphoric acid with methyl, ethyl and other alcohols are stable to alkali, as was shown by Lossen and Köhler in 1891.

In order to determine the composition of the natural organic phosphorus compounds they are hydrolysed by heating with acid at a high temperature and very frequently under pressure. The hydrolysis of glycerophosphoric acid by acids has recently been investigated by Malengreau and Prigent [1911] who find that the decomposition proceeds according to the laws of a monomolecular reaction and that it corresponds to the hydrolysis of ethyl phosphoric acid and other esters of phosphoric acid studied by Cavalier [1899].

There appear to be no further data concerning the hydrolysis of the natural organic phosphorus compounds by acids and alkalies. The statement is usually made that they are decomposed very easily and that loss, due to hydrolysis, occurs in their isolation from tissues when the solutions are being concentrated. More definite information as to their hydrolysis in solution,

both alone and in the presence of acid and alkali, is therefore required. Such data are not only of use in their preparation, but may also serve for the elucidation of their constitution. More practical knowledge concerning the hydrolysis of phytic acid by acids was required for the estimation of inorganic phosphoric acid in the presence of phytic acid and for the comparison of the hydrolysis of the various compounds with the hydrolysis by enzymes (see preceding paper). The experiments have been carried out for these purposes and no attempt has been made to ascertain the kinetics of the reaction.

EXPERIMENTAL.

Dilute acid and dilute alkali of concentrations varying from normal to twice normal have been used. The hydrolysis by acids was carried out in glass vessels, that by alkali in copper vessels. Porcelain vessels were as unsuitable as glass owing to the solvent action of the alkali upon the glaze. The copper vessels were also attacked by the alkali: the solution generally contained dissolved copper as shown by the blue-green colour: the copper compound was precipitated on neutralising with acid and a colourless filtrate was obtained. The solution of the organic phosphorus compound was either mixed with the acid or alkali and samples of equal volume were placed in separate vessels which were maintained at the desired temperature, or the entire mixture was placed in a thermostat. The vessel was then removed, cooled to room temperature and samples taken for analysis.

The same preparations of glycerophosphoric acid, phytic acid, ethyl phosphoric acid and the same trypsin digest were used as described in the previous paper: hexosephosphoric acid was again kindly placed at my disposal by Dr Young: the nucleic acid was again prepared for me by Miss Skelton by Kossel's method and Mr Page gave me a further quantity of hydroxymethyl-phosphinic acid.

The analyses of phosphoric acid were made by precipitation with ammonium magnesium citrate and conversion into magnesium pyrophosphate. Total phosphoric acid in the solution was estimated in a separate sample by Neumann's method. All results were then calculated out in terms of P_2O_5 for the same volume.

As mentioned in the previous paper, inorganic P_2O_5 is not precipitated in the presence of phytic acid. Inorganic P_2O_5 was therefore estimated by precipitation with ammonium molybdate. The hydrolysis by acids was carried out with nitric acid. In most experiments the phytin was dissolved in the nitric acid; there was no necessity to remove the calcium as its presence in the solution does not interfere with the precipitation of the

ammonium phosphomolybdate. When alkali was used the cooled sample was neutralised with nitric acid, an equal volume of 2N nitric acid, and then 20–30 c.c. of 10 per cent. ammonium molybdate solution added; the precipitation began almost immediately and in 24 hours the solution was quite colourless. The precipitate was then filtered off and estimated by the Neumann method by solution in seminormal caustic soda and titration with seminormal sulphuric acid after boiling off the ammonia. The analytical figures obtained are evidence that inorganic P_2O_5 can be estimated by this method in the presence of phytic acid. Details concerning the inhibition of the precipitation of inorganic P_2O_5 by ammonium magnesium citrate in presence of phytic acid and the estimation by ammonium molybdate at room temperature will be given in a later paper dealing with the analysis of phytin.

The details of these experiments are:

I. *Hydrolysis of Glycerophosphoric Acid.*

(a) *By Acid.*

i. By $\frac{N}{4}$ H_2SO_4 at 86° C.

20 c.c. glycerophosphoric acid diluted to 500 c.c. with water. 50 c.c. samples + 50 c.c. $\frac{N}{2}$ H_2SO_4 kept at 86° C.

	P_2O_5 in gm.
At commencement	0·0069
After 3 hours ...	0·0092
,, 6 ,, ...	0·0112
,, 15 ,, ...	0·0391
,, 24 ,, ...	0·0494
,, 2 days ...	0·0756
,, 3 ,, ...	0·0855
,, 6 ,, ...	0·0906
Total	0·2675

ii. By $\frac{N}{4}$ H_2SO_4 at 92° C.

20 c.c. glycerophosphoric acid diluted to 500 c.c. with water. 50 c.c. samples + 50 c.c. $\frac{N}{2}$ H_2SO_4 kept at 92° C.

	P_2O_5 in gm.
At commencement	0·0102
After 1 day ...	0·0547
,, 2 days ...	0·0683
,, 3 ,, ...	0·0928
,, 4 ,, ...	0·0998
,, 6 ,, ...	0·1578
,, 10 ,, ...	0·2629
Total	0·2587

(b) *Autohydrolysis.*

i. At 95° C.

20 c.c. glycerophosphoric acid diluted to 500 c.c. 50 c.c. samples kept at 95° C.

	P_2O_5 in gm.
At commencement	0·0074
After 2 hours ...	0·0114
,, 4 ,, ...	0·0154
,, 6 ,, ...	0·0179
,, 8 ,, ...	0·0194
,, 16 ,, ...	0·0342
Total	0·2612

ii. At 75° C.

25 c.c. glycerophosphoric acid diluted to 500 c.c. and kept at 75° C.

	P_2O_5 in gm.
At commencement	0·0157
After 2 days ...	0·0216
,, 3 ,, ...	0·0281
,, 4 ,, ...	0·0341
,, 5 ,, ...	0·0404
,, 6 ,, ...	0·0458
,, 7 ,, ...	0·0512
,, 9 ,, ...	0·0623
Total	0·1374

(c) By Alkali.

i. By $\frac{N}{1}$ NaOH at 95° C.

25 c.c. glycerophosphoric acid diluted with 700 c.c. $\frac{N}{1}$ NaOH.

P$_2$O$_5$ in gm.

At commencement	0·0137
After 2 days ...	0·0144
,, 3 ,, ...	0·0118
,, 5 ,, ...	0·0150
,, 7 ,, ..	0·0195
.. 10 ..	0·0179

Total 0·2194

ii. By $\frac{2N}{1}$ NaOH at 75° C.

6 gm. sodium glycerophosphate dissolved in 550 c.c. $\frac{2N}{1}$ NaOH (5 c.c. = 20·1 c.c. $\frac{N}{2}$ H$_2$SO$_4$) and kept at 75° C.

P$_2$O$_5$ in gm.

At commencement		——
After 2 days	...	0·0011
.. 4 ..	...	0·0023
,, 8 ,,	...	0·0010
,, 16 ,,	...	0·0018
,, 32 ,,	...	0·0033
,, 81 ,,	...	0·0078

Total 0·1750

II. Hydrolysis of Ethyl Phosphoric Acid.

(a) By acid $\left(\frac{N}{1} HCl\right)$ at 75° C.

300 c.c. sodium ethyl phosphate solution + 300 c.c. $\frac{2N}{1}$ HCl.

P$_2$O$_5$ in gm.

At commencement		0·0001
After 8 hours	...	0·0003
,, 1 day	...	0·0013
,, 2 days	...	0·0031
,, 3 ,,	...	0·0045
,, 4 ,,	...	0·0065
,, 5 ,,	...	0·0077
,, 6 ,,	...	0·0091
,, 8 ,,	...	0·0115
,, 11 ,,	...	0·0146
,. 16 .,	...	0·0204
,, 20 ,,	...	0·0240

Total 0·0469

(b) By alkali $\left(\frac{N}{1} NaOH\right)$ at 75° C.

300 c.c. sodium ethyl phosphate solution + 300 c.c. $\frac{2N}{1}$ NaOH.

P$_2$O$_5$ in gm.

At commencement		0
After 1 day	...	0
,, 5 days	...	0
,, 13 ,,	...	0
,, 33 ,,	...	0

Total 0·0926

(c) Autohydrolysis at 75° C. 200 c.c. ethyl phosphoric acid solution.

P$_2$O$_5$ in gm.

At commencement		0·0002
After 1 day	...	0·0018
,, 2 days	...	0·0033
,, 4 ,,	...	0·0064
,, 6 ,,	...	0·0094
,, 50 ,,	...	0·0526

Total 0·0746

III. Hydrolysis of Phytic Acid.

(a) By Acid.

i. By $\frac{N}{1}$ HNO$_3$ at 37° C.

125 c.c. phytic acid solution + 125 c.c. $\frac{2N}{1}$ HNO$_3$.

P$_2$O$_5$ in gm.

At commencement		0·0127
After 1 day	...	0·0144
,, 2 days	...	0·0142
,, 5 ,,	...	0·0134

Total 0·2156

ii. By $\frac{2N}{1}$ HNO$_3$ at 37° C.

1 gm. phytin dissolved in 250 c.c. $\frac{2N}{1}$ HNO$_3$.

P$_2$O$_5$ in gm.

At commencement		0·0055
After 2 days	...	0·0062
.. 5 ..	...	0·0062
.. 8 ..	...	0·0066

Total 0·0976

iii. By $\frac{N}{1}$ HNO$_3$ at 65° C.

1 gm. phytin dissolved in 130 c.c. H$_2$O + 130 c.c. $\frac{2N}{1}$ HNO$_3$.

	P$_2$O$_5$ in gm.
At commencement	0·0051
After 1 hour ...	0·0056
,, 2 hours ...	0·0057
,, 4 ,, ...	0·0054
,, 6 ,, ...	0·0058
Total 0·0938	

iv. By $\frac{2N}{1}$ HNO$_3$ at 64° C.

1 gm. phytin dissolved in 260 c.c. $\frac{2N}{1}$ HNO$_3$.

	P$_2$O$_5$ in gm.
At commencement	0·0046
After 15 mins. ...	0·0051
,, 30 ,, ...	0·0053
,, 1 hour ...	0·0053
,, 2 hours ...	0·0057
Total 0·0913	

v. By $\frac{2N}{1}$ HNO$_3$ at 64° C.

1 gm. phytin dissolved in 260 c.c. $\frac{2N}{1}$ HNO$_3$.

	P$_2$O$_5$ in gm.
At commencement	0·0049
After 3 hours ...	0·0056
,, 6 ,, ..	0·0060
,, 9 ,, ...	0·0061
,, 24 ,, ...	0·0065
Total 0·1040	

vi. By $\frac{N}{1}$ HNO$_3$ at 75° C.

1 gm. phytin dissolved in 260 c.c. $\frac{N}{1}$ HNO$_3$.

	P$_2$O$_5$ in gm.
At commencement	0·0063
After 1 day ...	0·0134
,, 2 days ...	0·0232
,, 4 ,, ...	0·0280
,, 8 ,, ...	0·0439
Total 0·0926	

vii. By $\frac{N}{1}$ HNO$_3$ at 75° C.

1 gm. phytin dissolved in 260 c.c. $\frac{N}{1}$ HNO$_3$.

	P$_2$O$_5$ in gm.
At commencement	0·0056
After 1 day ...	0·0116
,, 2 days ...	0·0165
,, 4 ,, ...	0·0259
,, 8 ,, ...	0·0436
Total 0·1014	

viii. By $\frac{2N}{1}$ HNO$_3$ at 75° C.

2·5 gm. phytin dissolved in 500 c.c. $\frac{2N}{1}$ HNO$_3$.

	P$_2$O$_5$ in gm.
At commencement	0·0077
After 1 day ...	0·0216
,, 2 days ...	0·0349
,, 4 ,, ...	0·0571
,, 6 ,, ...	0·0777
,, 8 ,, ...	0·0976
,, 9 ,, ...	0·1040
,, 11 ,, ...	0·1147
,, 12 ,, ...	0·1192
,, 14 ,, ...	0·1243
,, 15 ,, ...	0·1255
,, 16 ,, ...	0·1274
,, 17 ,, ...	0·1312
Total 0·1471	

(b) *By Alkali.*

By $\frac{N}{1}$ NaOH at 75° C.

300 c.c. sodium phytate solution + 300 c.c. $\frac{2N}{1}$ NaOH.   (5 c.c. = 9·7 c.c. $\frac{N}{2}$ H$_2$SO$_4$.)

P$_2$O$_5$ in gm.

At commencement	0·0093
After 1 day ...	0·0089
,, 2 days ...	0·0091
,, 4 ,, ...	0·0079
,, 8 ,, ...	0·0094
,, 16 ,, ...	0·0106
,, 32 ,, ...	0·0119
Total 0·1407	

(c) *Autohydrolysis at* 75° C.

600 c.c. phytic acid solution.

P$_2$O$_5$ in gm.

At commencement	0·0034
After 1 day ...	0·0071
,, 2 days ...	0·0112
,, 3 ,, ...	0·0151
,, 4 ,, ...	0·0191
,, 5 ,, ...	0·0235
,, 7 ,, ...	0·0314
Total 0·0527	

IV. *Hydrolysis of Hexosephosphoric Acid.*

(a) By acid $\left(\frac{N}{1} \text{HCl}\right)$ at 75° C.

100 c.c. hexosephosphoric acid solution neutralised with NaOH + 100 c.c. $\frac{2N}{1}$ HCl. (5 c.c. = 11·2 c.c. $\frac{N}{2}$ NaOH.)

P$_2$O$_5$ in gm.

At commencement	0·0034
After 7 hours ...	0·0348
,, 1 day ...	0·0443
,, 2 days ...	0·0480
,, 3 ,, ...	0·0486
,, 5 ,, ...	0·0508
,, 8 ,, ...	0·0508
Total 0·0527	

(b) By alkali $\left(\frac{1\cdot5\,N}{1} \text{NaOH}\right)$ at 75° C.

40 c.c. hexosephosphoric acid solution + 500 c.c. $\frac{2N}{1}$ NaOH. (5 c.c. = 16·5 c.c. $\frac{N}{2}$ H$_2$SO$_4$.)

P$_2$O$_5$ in gm.

At commencement	0·0136
After 1 day ...	0·1610
,, 2 days ...	0·1598
,, 4 ,, ...	0·1641
Total 0·1978	

(c) Autohydrolysis at 75° C.

50 c.c. hexosephosphoric acid solution + 220 c.c. water. (5 c.c. = 0·2 c.c. $\frac{N}{2}$ NaOH.)

P$_2$O$_5$ in gm.

At commencement	0·0037
After 1 day ...	0·0494
,, 2 days ...	0·0698
,, 3 ,, ...	0·0791
,, 18 ,, ...	0·1152
Total 0·1243	

V. *Hydrolysis of Nucleic Acid.*

(a) By acid $\left(\frac{N}{1} \text{HCl}\right)$ at 75° C.

3·5 gm. thymus nucleic acid dissolved in 200 c.c. H$_2$O + 10 c.c. $\frac{2N}{1}$ NaOH ; 210 c.c. $\frac{2N}{1}$ HCl then added; the precipitate went into solution in 15 minutes at 75° C.

P$_2$O$_5$ in gm.

At commencement	——
After 4 hours ...	0·0250
,, 1 day ...	0·0429
,, 2 days ...	0·0545
,, 3 ,, ...	0·0612
,, 4 ,, ...	0·0968
,, 8 ,, ...	0·1106
Total 0·1103	

(b) By alkali $\left(\frac{N}{1} \text{NaOH}\right)$ at 75° C.

6 gm. nucleic acid dissolved in 600 c.c. $\frac{N}{1}$ NaOH. (5 c.c. = 10 c.c. $\frac{N}{2}$ H$_2$SO$_4$.)

P$_2$O$_5$ in gm.

At commencement	0
After 1 day ...	0·0040
,, 2 days ...	0·0106
,, 4 ,, ...	0·0179
,, 16 ,, ...	0·0369
,, 32 ,, ...	0·0510
,, 76 ,, ...	0·0990
Total 0·1128	

(c) By alkali $\left(\dfrac{N}{1}\ \text{NaOH}\right)$ at 70° C.

4 gm. nucleic acid dissolved in 200 c.c. H_2O + 200 c.c. 2N NaOH.

P_2O_5 in gm.

At commencement	...	...		0·0020
After 1 day	...	...	...	0·0108
,, 2 days	...	...	...	0·0145
,, 4 ,,	...	...	...	0·0236
,, 9 ,,	...	...	...	0·0305
,, 22 ,,	...	...	..	0·0447

Total 0·0951

VI. *Hydrolysis of Phosphoprotein by Alkali.*

i. 200 c.c. trypsin digest of caseinogen + 50 c.c. $\dfrac{2N}{1}$ NaOH. (= 1·6 per cent. NaOH.)

ii. 175 c.c. trypsin digest of caseinogen + 25 c.c. $\dfrac{2N}{1}$ NaOH. (= 1 per cent. NaOH.)

iii. 180 c.c. trypsin digest of caseinogen + 20 c.c. $\dfrac{2N}{1}$ NaOH. (= 0·8 per cent. NaOH.)

P_2O_5 in gm.

At commencement		0·0194
After 1 day	...	0·0320
,, 2 days	...	0·0321
,, 3 ,,	...	0·0328

Total 0·0342

P_2O_5 in gm.

At commencement		0·0199
After 1 day	...	0·0327
,, 2 days	...	0·0342

Total 0·0380

P_2O_5 in gm.

At commencement		0·0209
After 1 day	...	0·0272
,, 2 days	...	0·0300
,, 4 ,,	...	0·0334

Total 0·0406

VII. *Hydrolysis of Hydroxymethyl-phosphinic Acid by Acid.*

130 c.c. sodium hydroxymethyl-phosphinate solution + 130 c.c. $\dfrac{2N}{1}$ HNO_3. (5 c.c. = 10·3 c.c. $\dfrac{N}{2}$ NaOH.)

P_2O_5 in gm.

At commencement	...	...		0·0006
After 1 day	...	...	...	0·0004
,, 2 days	...	...	...	0·0006
,, 4 ,,	...	...	...	0·0006
,, 8 ,,	...	...	...	0·0018

Total 0·1179

Glycerophosphoric acid is slowly hydrolysed by dilute acid, complete separation of the phosphoric acid requiring 10 days at 92° C. The glycerol, which is formed, was isolated in another experiment by removing the sulphuric and phosphoric acids by baryta, concentrating and distilling *in vacuo*: the glycerol distilled at 162° C. at 10–15 mm. pressure and a yield of 44 per cent. was obtained. Autohydrolysis is considerably slower; one-eighth of the glycerophosphoric acid was decomposed in 16 hours at 95° C., and only one-half in 9 days at 75° C. It is not hydrolysed by alkali ; the slight increase in the amount of inorganic phosphate observed after 81 days by the action of twice normal caustic soda was due to slight evaporation through the cork ; the total P_2O_5 at the end of the period being 0·1750 gm., which is a little greater than that at the beginning—0·1509 gm.

Glycerophosphoric acid is stable to alkali like ethyl phosphoric acid; Lossen and Köhler's result has been confirmed and autohydrolysis and acid hydrolysis of ethyl phosphoric acid have been carried out at 75° C. for comparison with the other compounds. Autohydrolysis of ethyl phosphoric acid was not complete in 50 days and the hydrolysis by acid seems to be slower than in the case of glycerophosphoric acid.

Phytic acid is the most stable of the organic phosphorus compounds. At 37° C. dilute acid effected no hydrolysis; at 64° C. a slight hydrolysis could be detected in 24 hours; at 75° C. normal nitric acid separated about half of the phosphorus as inorganic phosphate in 8 days. Complete hydrolysis was not effected by twice normal nitric acid in 17 days at the same temperature. Autohydrolysis at 75° C. resulted in the splitting off of about half the phosphoric acid in 7 days.

Phytic acid is also quite stable to alkali; the slight increase in the amount of inorganic phosphate noted after 32 days by normal alkali at 75° C. is due to concentration of the solution by evaporation through the cork.

Hexosephosphoric acid is easily hydrolysed by both acid and alkali. Complete hydrolysis was effected by normal hydrochloric acid in 3–5 days, the greater part of the phosphoric acid being separated in 1 day. Autohydrolysis at 75° C. was much slower than acid hydrolysis; half the phosphoric acid was separated in 2 days; complete hydrolysis occurred in about 18 days. Dilute alkali produced complete hydrolysis in 1 day. In all the experiments a small quantity of organic phosphorus remained undecomposed: it is very probable that another organic phosphorus compound was present as impurity in the solution.

Nucleic acid is hydrolysed by both acid and alkali. Normal hydrochloric acid effected complete hydrolysis in 8 days at 75° C. With normal caustic soda a slow hydrolysis was observed: about one-third of the phosphoric acid was split off in 8 days, about one-half in 32 days; even after 76 days a small amount of organic phosphorus remained in solution.

The organic phosphorus compound which remains in a prolonged tryptic digest of caseinogen is also completely hydrolysed by 1 per cent. caustic soda; there is no difference in its behaviour from that of caseinogen; the former observation of Plimmer and Bayliss [1906] was incorrect.

Hydroxymethyl-phosphinic acid is not hydrolysed by dilute acid at 75° C.
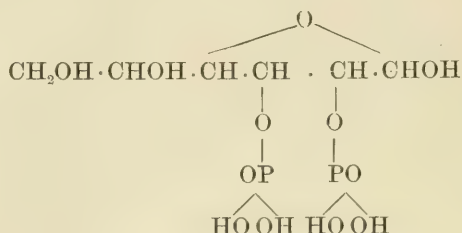
Summary and Conclusion.

Ethyl phosphoric acid, glycerophosphoric acid and phytic acid are hydrolysed by acid, but are stable to alkali. Stability to alkali is therefore a property of the esters of phosphoric acid.

Hexosephosphoric acid and phosphoprotein are so different in their behaviour to alkali from the above three compounds that some difference in their constitution from that of the esters must exist.
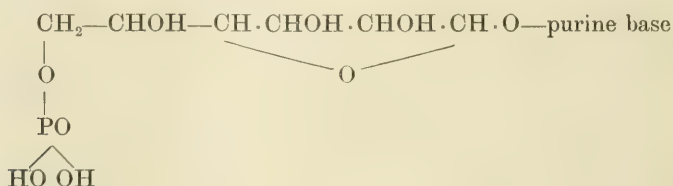
It is not known how the phosphoric acid is combined in phosphoprotein, but it is probably united with one of the amino-acids.

Hexosephosphoric acid reduces Fehling's solution which points to the presence of the functional aldehyde or ketone group in the molecule.

The phosphoric acid radicles are most probably combined with two of the hydroxyl groups leaving the reducing group free. The action of the alkali will destroy this grouping and the whole carbohydrate molecule will be decomposed leaving the phosphoric acid:

$$\underset{\underset{\underset{\underset{\text{HO OH HO OH}}{\overset{\wedge\quad\wedge}{}}}{\underset{\text{OP}\quad\text{PO}}{|\quad\quad|}}}{\underset{\text{O}\quad\text{O}}{|\quad\quad|}}{\overset{\overset{\text{O}}{\diagup\quad\diagdown}}{\text{CH}_2\text{OH}\cdot\text{CHOH}\cdot\text{CH}\cdot\text{CH}\quad\cdot\quad\text{CH}\cdot\text{CHOH}}}$$

Nucleic acid, since it is hydrolysed like hexosephosphoric acid by both acid and alkali, seems to occupy an intermediate position between the stable esters and the very unstable hexosephosphoric acid. If the purine, or pyrimidine, base be attached to the functional aldehyde group in the same way as the alcohols in the glucosides, the action of alkali may be to destroy the purine base leaving the aldehyde group for decomposition of the molecule, and phosphoric acid will remain. A formula such as

$$\underset{\underset{\underset{\text{HO OH}}{\overset{\wedge}{}}}{\underset{\text{PO}}{|}}}{\underset{\text{O}}{|}}{\text{CH}_2\text{—CHOH—CH}\cdot\text{CHOH}\cdot\text{CHOH}\cdot\text{CH}\cdot\text{O—purine base}}$$

would explain the slow decomposition by alkali.

REFERENCES.

Cavalier (1899), *Ann. Chim. Phys.* [7], **18**, 449.
Lossen and Köhler (1891), *Annalen*, **262**, 209.
Malengreau and Prigent (1911), *Zeitsch. physiol. Chem.* **73**, 68.
Plimmer and Bayliss (1906), *J. Physiol.* **33**, 439.
Plimmer and Scott (1908), *J. Chem. Soc.* **93**, 1699.

VI. THE NITROGENOUS CONSTITUENTS OF LIME-JUICE.

By CASIMIR FUNK,

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(Preliminary Communication.)

From the Biochemical Department, Lister Institute of Preventive Medicine.

In connection with the work previously published on beri-beri [Funk, 1911, 1912, 1], in which the physiological importance of certain nitrogenous substances, belonging most probably to the pyrimidine group, was demonstrated, a similar fractionation of lime-juice was undertaken for the investigation of scurvy.

From the classical work of Holst and Fröhlich [1907, 1912] on scurvy we know that this disease has a close analogy to beri-beri and is also due to a lack of some substance in the food. They specially advanced our knowledge of this disease by inducing in animals (rabbits and guinea-pigs) a disease which possesses a great similarity in symptoms to human scurvy. They produced the experimental scurvy by an exclusive diet of oats or autoclaved vegetables and were able to prevent it by an addition to this diet of fresh vegetable-juice, unboiled milk or lime-juice. Lime-juice itself has been known for many years as an excellent remedy for scurvy.

Some time ago I was able to show [1912, 2] that lime-juice contains an anti-neuritic substance curing polyneuritis in birds, produced by an exclusive diet of polished rice, and a substance, present in another fraction, which is only capable of prolonging the life of these birds. Further experiments have shown that pyrimidine substances in general possess this life-prolonging action and a paper on this subject will shortly appear. No anti-scorbutic substance however was obtained from lime-juice.

I had at first intended to try each fraction, obtained during this

investigation, on guinea-pigs fed on oats, but in this I found considerable difficulties. The animals refused all food after a period of three weeks and even an addition of freshly made potato-juice or lime-juice was not able to save them from starvation. The same negative result was obtained with fractions of lime-juice which might have been expected to contain the anti-scorbutic substance in a more concentrated form.

The only positive results were obtained with milk, 50 c.c. daily of unboiled milk in addition to the oats diet preventing loss of weight and onset of scurvy. Even after the elimination of caseinogen and the other proteins present in milk, the latter keeps its preventive power. In doing this extreme care must be taken not to destroy the substance, and all boiling must be carefully avoided. The caseinogen is precipitated by addition of acetic acid at 50° and lactalbumin by dialysed iron-solution, kaolin however carries the substance down. The solution is then concentrated by freezing it and taking the supernatant liquid. This subject is being further investigated. This unstable character of the anti-scorbutic substance renders it unlikely that the usual method of separation, with the unavoidable use of alkali, would lead to its isolation. Although no anti-scorbutic substance was obtained from lime-juice, several new compounds have been detected and are described below.

Besides a substance, which apparently belongs to the terpene group and which has only been analysed and not further investigated, as having no direct connection with the subject, three nitrogenous substances have been isolated. One belongs to the purine group, the second to the pyrimidine group and the third to the choline group. The small yield obtained did not allow however of a complete investigation, so that this paper must be regarded as preliminary.

EXPERIMENTAL.

407 litres of commercial lime-juice were precipitated in portions of 15 litres each with 2800 grms. of neutral lead acetate. The bulky precipitate was filtered off on a large Buchner funnel and the residue pressed out in a hydraulic press. The combined filtrates were freed from the excess of lead, first by an addition of sulphuric acid, then by sulphuretted hydrogen, and were then concentrated *in vacuo* at low temperature. During the distillation crystals separated out, which were filtered off, and weighed when dry 258 grms. These were recrystallised from 20 % acetic acid and 162 grms. were obtained in needles. The substance proved to be free from nitrogen and was recrystallised for analysis once more from water. It is

nearly insoluble in all other solvents, except acetic acid, and is volatile with steam; M.P. 97°–100° (uncorr.).

0·1357 grm. gave   0·3400 CO_2 and 0·1312 H_2O.
Found   68·33 % C ;   10·73 % H.
Calc. for $C_{13}H_{24}O_3$; 68·42 % C ;   10·52 % H.

This substance probably belongs to the terpene group; it possesses a very characteristic agreeable smell when heated. The nature of this compound, which has no direct relationship with the scurvy problem, was not further investigated.

To the filtrate from this substance enough sulphuric acid was added to make a 5 % solution and then a 50 % solution of phospho-tungstic acid until precipitation was complete. The precipitate obtained amounted to nearly 12 kg., and consisted chiefly of potassium salt.

Investigation of the Phospho-tungstate Precipitate.

The precipitate was decomposed in portions of 1 kg. each with $2\frac{1}{2}$ kg. baryta in a mortar and the mixture was shaken on the shaking machine for one hour. The precipitate was then filtered off, suspended in water and again shaken. The excess of baryta was eliminated from the combined filtrates by the careful addition of dilute sulphuric acid. The solution obtained was then neutralised with nitric acid, as it was strongly alkaline, and evaporated *in vacuo* to about two litres. In this solution the usual fractionation with silver nitrate was performed and three fractions were obtained. The first fraction came down on addition of a silver nitrate solution and contained the substances of the purine group. This precipitate was filtered off and to the filtrate a saturated solution of silver nitrate was added until a drop of the liquid gave with a cold baryta solution a brown precipitate which indicates an excess of silver. A saturated aqueous baryta solution was then added until a drop of the clear liquid gave only a small precipitate with silver nitrate and ammonia. The second precipitate consists of pyrimidine bases and substances of the histidine type. To the filtrate pulverised baryta was added until the whole of the silver was precipitated. The fraction thus obtained contains, in addition to traces of pyrimidine substances, compounds of the arginine group. The last filtrate contains substances of the choline group. All the fractions were investigated separately.

INVESTIGATION OF THE FIRST SILVER FRACTION.

The bulky precipitate obtained by a simple addition of silver nitrate was filtered off, well washed with water, suspended in water and the double silver nitrate salts converted into the silver salts by heating them on the water-bath with ammonia. The precipitate was filtered off and washed with water, until the filtrate was free from nitric acid. It was then suspended in water and decomposed with sulphuretted hydrogen. The solution on evaporation yielded 1 grm. of substance, which however contained a little ash. It was recrystallised from water and gave 0·7 grm. of crystals in the form of plates. After drying *in vacuo* at 110° they became brown at 240° and melted at 282° (corr.). The substance is precipitated by mercuric acetate and partially by gold chloride. It differs from the known purine bodies by the great solubility in hot water and the absence of all known reactions for these derivatives. Heated with nitric acid it becomes yellow, and brown when more strongly heated, no change of colouration being produced on the addition of alkali.

0·3855 grm. loses at 110° (*vacuo*) 0·0190 grm. H_2O
0·1176   ,,   requires 32·7 c.c. N/10 H_2SO_4
0·1372   ,,   ,,   38 c.c.   ,,   ,,
0·1226   ,,   gave 0·1788 CO_2 and 0·0196 H_2O

Calc. for $C_6H_7O_2N_5$ (Mol. wt.=181)	Found	
C 39·78 %	39·77	
H 3·86 %	3·55	
N 38·67 %	38·77	38·92
½ Mol. H_2O 4·73 %	4·92	

INVESTIGATION OF THE SECOND SILVER FRACTION.

The second silver nitrate precipitate was filtered off, washed well with water and decomposed with sulphuretted hydrogen. In the filtrate the last traces of baryta were taken out with a very dilute solution of sulphuric acid and the solution was evaporated *in vacuo*. As the residue did not deposit any crystals, the solution was precipitated with mercuric sulphate. The solution obtained by decomposition of the precipitate showed no tendency to crystallisation, but on addition of a picric acid solution an oil separated, which was decanted and dissolved in acetone. After evaporation a solid powder was obtained which amounted to 0·35 grm. This was recrystallised from a mixture of alcohol and acetone and yielded about 0·1 grm. of brown prisms, which decomposed at 205°–210°. An amount of this fraction

corresponding to 0·01 grm. of the picrate was decomposed and given to a polyneuritic pigeon which showed a considerable improvement and died only after four days.

The mercuric sulphate filtrate, freed from mercury and sulphuric acid, was evaporated *in vacuo* and the residue left in a desiccator after addition of alcohol. After a few days a colourless substance separated out which amounted to 0·7 grm. After recrystallisation from dilute alcohol 0·25 grm. was obtained in the form of microscopical spherolites, which after drying at 110° *in vacuo*, melted and decomposed at 188°–189°.

0·0958 grm. subst. required 7·5 c.c. N/10 H_2SO_4; 10·96 % N.
0·1034 ,, gave 0·1625 CO_2 and 0·0536 H_2O; 42·86 % C; 6·83 % H.
Calc. for $C_9H_{18}O_6N_2$ (Mol. wt.=250) 43·2 % C; 7·2 % H and 11·2 % N.

The third silver fraction yielded but little substance. On evaporation of the solution obtained by decomposing the precipitate no crystalline compound separated out. Picric acid solution however gave a precipitate which after standing formed crystalline, yellow spherolites, which began to decompose at 260°; yield 0·1 gr.

INVESTIGATION OF THE SILVER NITRATE FILTRATE.

Of this filtrate, which amounted to 4600 c.c., only 220 c.c. were taken for further investigation. To this liquid after elimination of silver and baryta, phospho-tungstic acid was added and 420 grms. of a dry precipitate were obtained, which was decomposed in the ordinary way. The solution of the bases belonging to the choline group was neutralised with hydrochloric acid and evaporated *in vacuo* to dryness. To get rid of the last traces of water the residue was redissolved in alcohol and the alcohol evaporated *in vacuo*. Finally an alcoholic solution was made and an alcoholic solution of sublimate was added as long as a precipitate was formed. This was recrystallised from hot water with the addition of a little sublimate. It was found to be very slightly soluble in water. In this way 10·5 grms. of crystals were obtained in the form of cubes. These were redissolved in water and decomposed with sulphuretted hydrogen. The filtrate was evaporated *in vacuo* to dryness, the residue dissolved in alcohol and evaporated again. The alcoholic solution finally obtained was precipitated with an alcoholic platinic chloride solution, 1·3 grms. of a pale yellow precipitate being obtained which was recrystallised from dilute alcohol; 0·85 grm. was thus obtained in the form of needles, which, after drying at 110° *in vacuo*, melted at 220° (uncorr.). The substance gave the following figures on analysis, after being dried *in vacuo* at 110°.

0·1666 grm. gave 0·0451 grm. Pt
0·1622   ,,   required 4·2 c.c. N/10 H_2SO_4
0·2173   ,,   gave 0·2104 CO_2; 0·0878 H_2O ; 0·0589 Pt and 0·2586 AgCl.
Calc. for $(C_8H_{15}O_2NHCl)_2PtCl_4$ (Mol. wt. 724).   Found:

C : 26·52 ;	26·40
H : 4·42 ;	4·48
N : 3·86	3·62
Pt : 26·94 ;	27·07 ; 27·1
Cl : 29·55	29·44

On igniting the platinum salt a peculiar smell was noticed, which was quite different from that given by choline.

A substance of this formula, with similar properties (M.P. 219°), was described by v. Braun [1908] as the platinichloride of methylpiperidylacetic betaine. The substance is being further investigated.

REFERENCES.

v. Braun (1908), *Ber.* **41**, 2129.
Funk, Casimir (1911), *J. Physiol.* **43**, 395.
— (1912, 1), *J. Physiol.* **45**, 75.
— (1912, 2), *J. State Med.* **20**, 341.
Holst and Fröhlich (1907), *J. Hygiene*, **7**, 634.
—— (1912), *Zeitsch. Hyg. Infektionsk.* **72**, 1.

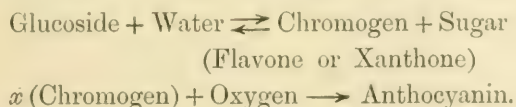# VII. THE FLOWER PIGMENTS OF *ANTIRRHINUM MAJUS*. I. METHOD OF PREPARATION.

By MURIEL WHELDALE,

Fellow of Newnham College, Cambridge.

I have previously [Wheldale, 1909, 2, 3; 1910, 2; 1911] made certain suggestions as to the nature of the chemical reactions involved in the formation of anthocyanin. There is little doubt that anthocyanin, as a collective term, in the same sense as protein, sugar, tannin, etc., includes many substances having in general similar properties, but differing among themselves as regards constitution. Evidence, from various sources, has led me to conclude that some anthocyanins may be derived from members of the groups of natural yellow colouring matters, known as the flavones and xanthones.

The yellow colouring matters are largely present in the plants as glucosides, some, or possibly all the hydroxyl groups, being replaced by sugar. I have suggested that the reactions involved in the formation of anthocyanin may be represented, in very general terms, as follows :—

$$\text{Glucoside} + \text{Water} \rightleftarrows \text{Chromogen} + \text{Sugar}$$
$$\text{(Flavone or Xanthone)}$$
$$x \, (\text{Chromogen}) + \text{Oxygen} \longrightarrow \text{Anthocyanin}.$$

The first reaction may be regarded as controlled by one or more glucoside-splitting enzymes and it is conceivable that specific enzymes may act on hydroxyl groups in different positions. When certain hydroxyl groups (position to be determined) are free from sugar, oxidation may take place at these points, or possibly condensation, or both, with the formation of anthocyanin. The residual hydroxyl groups in the anthocyanin molecule would probably be replaced by sugar and hence the anthocyanins would occur as glucosides. There is evidence that the second reaction may be brought about by an oxidase system [Wheldale, 1909, 3; 1910, 2; 1911; Keeble and Armstrong, 1912].

Some of the evidence for the above hypothesis is derived from results obtained in cross-breeding with varieties of *Antirrhinum majus* [Wheldale, 1907 ; 1909, 1 ; 1910, 1]. The wild type of this plant has magenta flowers, the colour due to anthocyanin. Among other varieties produced on cultivation are three without anthocyanin, i.e. ivory, yellow and white. Ivory and yellow contain pigments very probably of the flavone class. The white has no such pigment but carries other factors which apparently act on the yellow pigments to form anthocyanin. None of the three varieties alone can produce anthocyanin, but when ivory is crossed with white, a plant having magenta flowers is produced; when yellow with white, a plant having crimson (possibly a mixture of magenta and yellow) flowers.

With a view to testing this hypothesis I commenced a chemical investigation of the pigments of *Antirrhinum* and in the summer of 1911 I succeeded in finding a satisfactory method for obtaining the pigment in quantity in the solid state. Extractions were made from yellow and ivory separately, but all varieties containing magenta anthocyanin (i.e. ivory tinged with magenta, magenta and crimson) were extracted together. In this way all extracts contained mixtures of pigments, as will be seen below on referring to the varieties in detail. When attempting to purify the crude pigment later, I found difficulty in separating some of the pigments. Hence, when I again prepared pigment this year, the precaution was taken, in the case of certain varieties, of extracting portions only of the corolla, containing, to the best of my belief, either one pigment or two, which could be easily separated.

The method of preparation is as follows :—the flowers, picked off the spikes, are boiled with water in saucepans. The pigments are readily soluble in water and the extract is filtered through large funnels into lixiviating jars. The pigments are then precipitated as insoluble lead salts by adding solid crystalline lead acetate until no further precipitate appears. After standing a few hours the greater part of the liquid is decanted from the precipitates, which are then filtered through a large Buchner funnel attached to a filter pump. The lead salts are stirred up with 5–10 % sulphuric acid which decomposes the salts with formation of lead sulphate. The lead sulphate is filtered off and the filtrate contains the pigments as glucosides in dilute sulphuric acid solution. These solutions are boiled for several hours in two-litre Jena flasks fitted with simple tube condensers, care being taken to avoid concentration of the solution, since under such circumstances the pigment may become charred. Hydrolysis of the glucosides takes place during boiling and, on cooling and standing about 12 hours, the pigment, which is less soluble than the glucoside, is deposited. The deposit is separated by

filtering through as small a Buchner funnel as possible, a Geryk pump being used. After washing, the pigment is dried in a desiccator over sulphuric acid.

During the current year (1912), the following varieties were extracted. Excellent coloured figures of the varieties are given by Baur [1911]. Figures below refer to Baur's plate.

Ivory. (Fig. 3.) The corolla contains a pale yellow pigment (ivory) and a patch of deeper yellow pigment (yellow) on the lower lip. Extracts from the whole flower or from the lower lip gave an orange-yellow lead precipitate. From the separated upper lip, a canary-yellow lead precipitate. Pigment was prepared separately from both upper and lower lips.

Yellow. (Fig. 2.) The lips of the corolla contain yellow pigment which is chiefly confined to the epidermal cells. The inner tissues of the lips and the entire tube contain ivory pigment. Extracts gave an orange lead precipitate. Pigment was prepared separately from both upper and lower lips.

Ivory tinged with magenta. (Fig. 6.) Flower, ivory, with some development of magenta anthocyanin which is chiefly confined to the epidermis. Extracts gave a yellow-green lead precipitate. Pigment was prepared from entire flowers only and presumably contains ivory, yellow and magenta.

Magenta. (Fig. 7.) Magenta chiefly confined to epidermis; inner tissues contain ivory and there is a yellow patch on the lower lip. Extracts gave a deep green lead precipitate, some pigment apparently remaining in solution in the acetate, forming a deep green solution which is dichroic, red by transmitted and green by reflected light. Pigment was prepared separately from both upper and lower lips. The upper presumably contains magenta and ivory, the lower, yellow in addition.

Crimson. (Fig. 8.) Lips of the corolla are crimson and the tube magenta. It is at present uncertain whether the crimson colour is due to the presence of both yellow and magenta in the cells or to a distinct crimson pigment. Pigment was prepared from entire flowers only.

Rose doré. (Fig. 15.) Corolla contains a " red " anthocyanin [Wheldale 1909, 2]. The inner tissues, ivory, and there is a yellow patch on the lower lip. Extracts gave a reddish-green lead precipitate. Pigment was prepared from entire flowers only.

Bronze. (Fig. 17.) Bears the same relation to rose doré as crimson to magenta and it is again uncertain whether the colour is due to a mixture or to a separate pigment. Extracts gave a deep-red lead precipitate. Pigment was prepared from entire flowers only.

Methods of purification were first carried out on pigment obtained in 1911. As mentioned above, tinged ivory, magenta and crimson flowers had

then been extracted together. The crude pigment which presumably contained ivory, yellow and magenta was finely powdered thoroughly dried, placed in a Soxhlet thimble, and extracted first with warm ether in which the ivory and yellow pigments are soluble, though not readily. The anthocyanins, both magenta and red, are insoluble in ether.

From the ether extract two pigments were obtained by fractional crystallisation from alcohol and ethyl acetate. The less soluble pigment was taken to be ivory, the more soluble to be yellow. It is doubtful whether either pigment was obtained in the pure state.

Ivory pigment. This is readily soluble in alcohol and acetic acid, with more difficulty in ether and ethyl acetate, very slightly soluble in cold, more so in hot water; insoluble in chloroform and benzene. It crystallises from dilute alcohol in plates; M.P. 338°. It underwent combustion with difficulty in oxygen and did not give constant results for carbon. Tested by Zeisel's method for a methoxyl group, some silver iodide was obtained but only in such quantity as to indicate impurity. An acetyl derivative was prepared by boiling with acetic anhydride and pouring the product into sodium acetate solution. The product was purified with difficulty by crystallisation from ethyl acetate. The final product was pure white and crystallised in glistening needles; M.P. 182°. The combustion results were as follows:

$$C = 63{\cdot}19\,\% \qquad H = 4{\cdot}30\,\%$$
$$63{\cdot}21 \qquad\qquad 4{\cdot}22$$
$$62{\cdot}95 \qquad\qquad 4{\cdot}31$$

Of the known flavones, the ivory pigment bears most resemblance to apigenin in properties and acetyl derivative. Opinion is reserved as to its identity until further analyses have been made, especially with the ivory pigment (free from yellow) obtained from the upper lip of the ivory variety.

Yellow pigment. Crystallises in plates from dilute alcohol but was not obtained in the pure state; M.P. 290°–300°.

Magenta pigment. After extraction with ether for several weeks, the magenta pigment was obtained free from yellow. It crystallises, but not well, from a mixture of alcohol and ethyl acetate. It decomposes without melting when heated to 340°.

Other results obtained with magenta and yellow are reserved until further work has been done.

The crude pigment was extracted in the laboratory of the John Innes Horticultural Institution, Merton Park, Surrey. Large numbers of plants, the offspring of varieties originally used in breeding experiments, were grown

in the garden of the Institution. The yield of pigment was small and was not ascertained quantitatively because of the labour involved in weighing the flowers.

The work of purification and analysis, as far as it has gone, was carried out in the Biochemical Laboratory of the Institute of Physiology, University College, London. I am much indebted to Dr Aders Plimmer for kind help and suggestions.

REFERENCES.

Baur, E. (1911), " Einführung in die experimentelle Vererbungslehre." Tafel I.
Keeble, F. and Armstrong, E. F. (1912), *Proc. Roy. Soc.* **85 B**, 214.
Wheldale, M. (1907), *Proc. Roy. Soc.* **79 B**, 288.
—— (1909, 1), *Reports to Evolution Committee of the Royal Society*, Report **5**, 1.
—— (1909, 2), *Proc. Roy. Soc.* **81 B**, 44.
—— (1909, 3), *Proc. Cam. Phil. Soc.* **15**, 137.
—— (1910, 1), *Zeitsch. induktive Abstammungs- und Vererbungslehre*, **3**, 321.
—— (1910, 2), *Prog. Rei. Bot.* **3**, 457.
—— (1911), *J. Genetics*, **1**, 133.

VIII. THE DENSITY AND SOLUTION VOLUME OF SOME PROTEINS.

By HARRIETTE CHICK AND CHARLES JAMES MARTIN.

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In a previous communication dealing with the viscosity and other properties of caseinogen solutions [1912], we pointed out that there was a shrinkage in volume and corresponding increase in density when that protein formed colloidal solution. In order to investigate the point the method employed was to determine the density of the dry, powdered protein by weighing in benzene or some other indifferent fluid and to compare the value with that obtained by calculation from the observed density of a fairly concentrated solution. The latter value was found to be much greater than the former.

As far as we have been able to ascertain, this is the first attempt to determine the density of proteins with the exception of gelatin. Quincke [1903] found the specific gravity of a specimen of the latter to be 1·368, while Lüdekings [1888] had previously given the value of 1·412, and had shown that the density calculated from that of jellies (14 to 35 % gelatin) was as high as 1·9.

The increase in density when proteins are dissolved in water is an instance of the phenomenon constantly associated with colloidal solution of the lyophile or emulsoid character. The intimate association of the protein particles with water leads to a shrinkage in total volume, so that the increase in density is more properly regarded as an attribute of the whole system than of the protein alone. Analogous cases are those of gum-tragacanth and starch. In the case of the latter Rödewald [1897] found the density in the dry state and that calculated from that of a solution to be 1·38 and 1·49 respectively.

In our experiment with caseinogen, a sample of the pure, dry, powdered protein was prepared by acidifying a solution in dilute sodium hydroxide, washing and drying the precipitate, and grinding to a fine powder. A known weight was inserted in a pyknometer bottle, covered with benzene of known

density, and all the air expelled by careful heating *in vacuo*. The bottle was then filled up with benzene in a thermostat at 25° C. and weighed. The weight obtained was compared with that of the bottle filled with benzene at the same temperature, and the density of the powder calculated. The value obtained was 1·318 as the mean of two determinations (see Table I). A solution of caseinogen (7·85 %) in dilute sodium hydroxide gave a density of 1·0240, from which the density of sodium caseinogenate was calculated to be 1·42.

In order to compare this value with that obtained by direct determination, it is necessary to apply a small correction, seeing that the solution consisted of sodium caseinogenate. The ash present composed 4·331 % of the dry weight and, assuming this to be sodium carbonate, 2 % of the dry weight would consist of sodium, and the density, for comparison with that of the powdered caseinogen, should be reduced by that amount. After this correction has been made the caseinogen in solution is still seen to be denser than the solid caseinogen in the proportion of about 1·39 to 1·318.

In addition to caseinogen three other pure proteins have been similarly investigated, viz.:

Crystalline serum albumin, prepared from horse-serum by the method of Hopkins and Pinkus [1898].

Serum globulin (pseudo-globulin). Three different samples were investigated, obtained from horse-serum by different methods. I and II were prepared by repeated precipitation with half-saturated ammonium sulphate, the euglobulin present in the serum being separated by the subsequent dialysis; in the case of III the latter was removed by preliminary precipitation with saturated brine, after which the pseudo-globulin was precipitated from the warm diluted filtrate by adding anhydrous sodium sulphate until the concentration equalled 20 % Na_2SO_4. The precipitate was thoroughly washed with a solution of sodium sulphate of the same strength.

Crystalline egg-albumin, prepared from egg-white, also by the method of Hopkins and Pinkus.

All the proteins were thoroughly dialysed in presence of toluene and filtered, and concentrated solutions were finally obtained. Determinations were then made of the protein-content and the density. The latter were obtained by pyknometer readings, carried out usually at 15° C. or 20° C., the density of water at the same temperature being taken as unity for the purpose of calculation. It was found that the proportionality between the density of water and that of the protein solution was, within our error of experimentation, maintained between 15° C. and 25° C.

Dry specimens of the proteins were obtained by evaporating the solutions to dryness and grinding the residue to a fine powder. The evaporation and drying were carried out *in vacuo* at room-temperature, and were continued until the weight remained constant, an operation requiring 2–6 weeks. It was feared that, if the samples were dried at a high temperature, the denaturation of the proteins might introduce a source of error. This would not appear to be so, for, in the case of serum albumin, the densities of two samples dried at 100°–110° and 20° respectively, were not found to differ by any significant amount (Table I). The densities of the various powders were determined by weighing in benzene of known density (water at 4° C. = 1·00) at 25°, as described above.

TABLE I.

Density of Protein in solution compared with that in the solid state.

Protein	In solution			Dry State
	Concentration of protein % (by weight)	Density of the solution	Calculated density* of the protein	Density* of the protein
Caseinogen	7·85	1·0241	1·39†	1·318
Egg-albumin (crystalline) ...	14·6	1·0401	1·359	1·269‡
Serum-albumin (crystalline)	22·15	1·0647	1·378	1·275§
				1·281‡
Serum-globulin I	15·33	1·0428	1·365	1·279§
Serum-globulin II	16·35	1·0466	1·374	1·289‡
Serum-globulin III	11·05	1·0316	1·384	1·312‡

    * Density of water at 4° C. = 1·00.
    † Corrected for presence of sodium.
    ‡ Dried at room temperature *in vacuo.*
    § Dried at 105°—110° C.

The results are given in Table I and show that the density of albumin and globulin is also increased when in the state of colloidal solution, and to a higher degree (6·8%) than was found for caseinogen (5%). Egg- and serum-albumin have an almost identical density in the dry condition, viz. 1·269 and 1·281, and this is increased in the same proportion on entering solution, viz. to 1·359 and 1·378 respectively. The three samples of globulin give 1·293 as mean value when dry and 1·374 when in solution.

In Tables II and III are given the variation of the density of solutions of serum-albumin and serum-globulin with alteration in protein concentration. If the latter is plotted against the former, straight lines are obtained. The curve for caseinogen, on the other hand, shows a slight convexity which

is rather more than can be explained by our experimental error, the contraction on entering solution being proportionally greater for dilute solutions. This is shown in the third column of Table II, where the value of the density of caseinogen calculated from that of its solution is seen to decrease progressively from 1·465 to 1·412 as the concentration of the protein is increased. In the case of the serum proteins, however, the value remains constant (Table III).

TABLE II.

Density of Caseinogen (Na Caseinogenate) solutions of varying concentration.

Concentration of caseinogen $\%$ (by weight)	Density of the solution*	Calculated density of sodium caseinogenate
9·39	1·0283	1·412
8·33	1·0250	1·409
7·52	1·0232	1·424
6·05	1·0190	1·437
4·35	1·0140	1·460
2·173	1·0070	1·465
1·086	1·0033	†

* Compared with water = 1·00 at the same temperature, 15° C.
† Solution too dilute for trustworthy calculation.

TABLE III.

Density of solutions of Horse-Serum Proteins of varying concentration.

Protein			Concentration $\%$ (by weight)	Density of the solution*	Calculated density of the protein
Serum-globulin	...	...	15·33	1·0428	1·365
,,	...	...	10·32	1·0290	1·374
,,	...	...	6·916	1·0190	1·365
,,	...	...	3·478	1·0096	1·370
Serum-albumin	...	...	22·15	1·0647	1·378
,,	...	...	15·16	1·0440	1·382
,,	...	...	7·725	1·0220	1·381

* Compared with water = 1·00 at the temperature of expt.

SUMMARY.

A comparison has been instituted in case of four proteins, viz. caseinogen, egg- and serum-albumin, and serum globulin, between the density directly determined with dry specimens and that calculated from the specific gravity

of concentrated solutions. The latter is found to be 5–8 % in excess of the former, showing the extent of shrinkage in volume taking place when these proteins enter colloidal solution.

REFERENCES.

Chick and Martin (1912), *Zeitsch. Chem. Ind. Kolloide*, **11**, 102.
Hopkins and Pinkus (1898–1899), *J. Physiol.*, **23**, 130.
Lüdekings (1888), *Wied. Ann.* **35**, 552.
Quincke (1903), *Drude's Annalen*, **10**, 478.
Rödewald (1897), *Zeitsch. physikal. Chem.* **24**, 193.

IX. A NOTE CONCERNING THE INFLUENCE OF DIETS UPON GROWTH.

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In a recent paper Osborne and Mendel [1912, 1] have described certain experiments which seem to show that young animals (rats) can grow when fed upon artificial diets consisting of " purified " constituents alone.

These experiments would therefore indicate that the accessory factors of uncertain nature (complex lipoids or "vitamines" or "hormones") which others have believed to be necessary are not, as a matter of fact, indispensable. Such experimental results must give pause to those who like ourselves are engaged in an endeavour to separate, and identify more closely, the accessory substances referred to. But they are results which contradict what is now a considerable body of experience, and the experiments which yielded them seem to call for repetition.

These particular experiments were in a sense merely incidental to a wide enquiry, on the part of the authors quoted, into the nutritive efficiency of various proteins; and those which are of significance to the matter at issue (those, namely, which showed actual growth, and not maintenance alone) concerned only three animals [1912, 1, pp. 356, 358]. In a later paper [Osborne and Mendel, 1912, 2] the authors again refer incidentally to the subject and speak of having obtained " a considerable degree of success " by feeding in the absence of " the hypothetical organic hormones," etc. [1912, 2, p. 242].

But the weight-curves given in this paper show little more than maintenance of the animal, without growth; while, for some reason, the three experiments of the earlier paper which showed complete success in the promotion of vigorous growth are not further quoted. This success the authors attributed in the main to the fact that, in this dietary, salts were

supplied in a mixture made to imitate as exactly as possible the salts of milk ; but it is difficult to understand how animals so omnivorous as rats can depend for normal growth upon a very exact balance of particular acid and basic ions. In the experiments described by one of us in an earlier paper [Hopkins, 1912] the salts administered were obtained by carefully ashing a normal food mixture of proved efficiency. Upon an artificial diet containing these salts the animals did not grow ; but they grew at once when certain substances were added to the diet, some of these addenda being certainly incapable of supplying any deficiencies in the inorganic constituents of the original diet.

We have now fed a large number of rats upon the diet employed by Osborne and Mendel. The salt mixture as described by them was made with the greatest care, and all their directions for the preparation of the food mixture were exactly followed. But the protein and starch were thoroughly extracted with alcohol, and the lactose used was several times precipitated from its aqueous solution by the addition of alcohol. The methods of feeding were exactly those used in the paper by one of us already referred to, except that the food, being more fatty and coherent, was not mixed with water.

Twenty-four rats from various sources, of weights from 50 to 60 grms., were placed upon the mixture. Although the consumption of food was satisfactory, every rat, without exception, rapidly ceased to grow. In the greater number growth ceased as early as the sixth day, in some on the ninth, and in all before the fifteenth day. A comparatively brief period of maintained weight was then followed by a steady decline. In the case of eighteen of the animals the diet was administered up to the time of death, which, in all but four cases, occurred before the fortieth day.

To six of the above set of rats, after the decline in their weight had begun, 2 c.c. of milk per diem were given. An immediate betterment of the general condition of the animals followed ; growth was re-established and the health then maintained. In another experiment six rats were put upon Osborne and Mendel's diet, but were given milk from the first. In each case the animal grew.

We have spoken of the food consumption (which was carefully determined each day) as being satisfactory. It was smaller however than the consumption of the animals upon a somewhat different artificial dietary in the experiments described earlier by one of us. Its energy value, during the period which preceded actual loss of weight, was just under 40 calories per 100 grms. live weight, instead of over 50 calories. That the former value is nevertheless well in excess of the amount required for maintenance is shown by some, as

yet unpublished, experiments upon comparable animals made in the Cambridge Physiological Laboratory by Miss Hill.

When the small ration of milk was given each day in advance of feeding with the Osborne and Mendel diet the food consumption remained of the same order, and did not rise to the amount consumed by the rats in Hopkins' earlier experiments [1912]. The resulting growth though quite definite and steady was distinctly slower than in the experiments mentioned.

Our rats clearly behaved very differently from the three animals fed upon a similar diet by Osborne and Mendel. The difference we are unable to explain. Realising from previous experience how very small a remainder of the substances which are extracted by alcohol may leave an artificial dietary with some power of maintaining growth, and knowing that ether is a greatly inferior solvent for them, we fed rats upon the Osborne and Mendel mixture in which the protein (commercial casein) was extracted with ether only (as in one of Osborne and Mendel's experiments [1912, 1]; Curve 58, p. 358) and the lactose not crystallised from alcohol. We were unable to obtain growth however, though even this small difference in the diet appreciably lengthened the period during which the animals remained in health.

We do not in this note propose to publish the schedules of weights, etc. They will be given elsewhere at a later date. The purpose of the present note is to indicate that there is still reason for a continuance of the search for special accessory substances of potent influence upon growth. It should be pointed out that Osborne and Mendel themselves admit that such substances may exist.

REFERENCES.

Hopkins, F. G. (1912), *J. Physiol.* **44**, 425.
Osborne and Mendel (1912, 1), *Zeitsch. physiol. Chem.* **80**, 307.
Osborne and Mendel (1912, 2) *J. Biol. Chem.* **13**, 233.
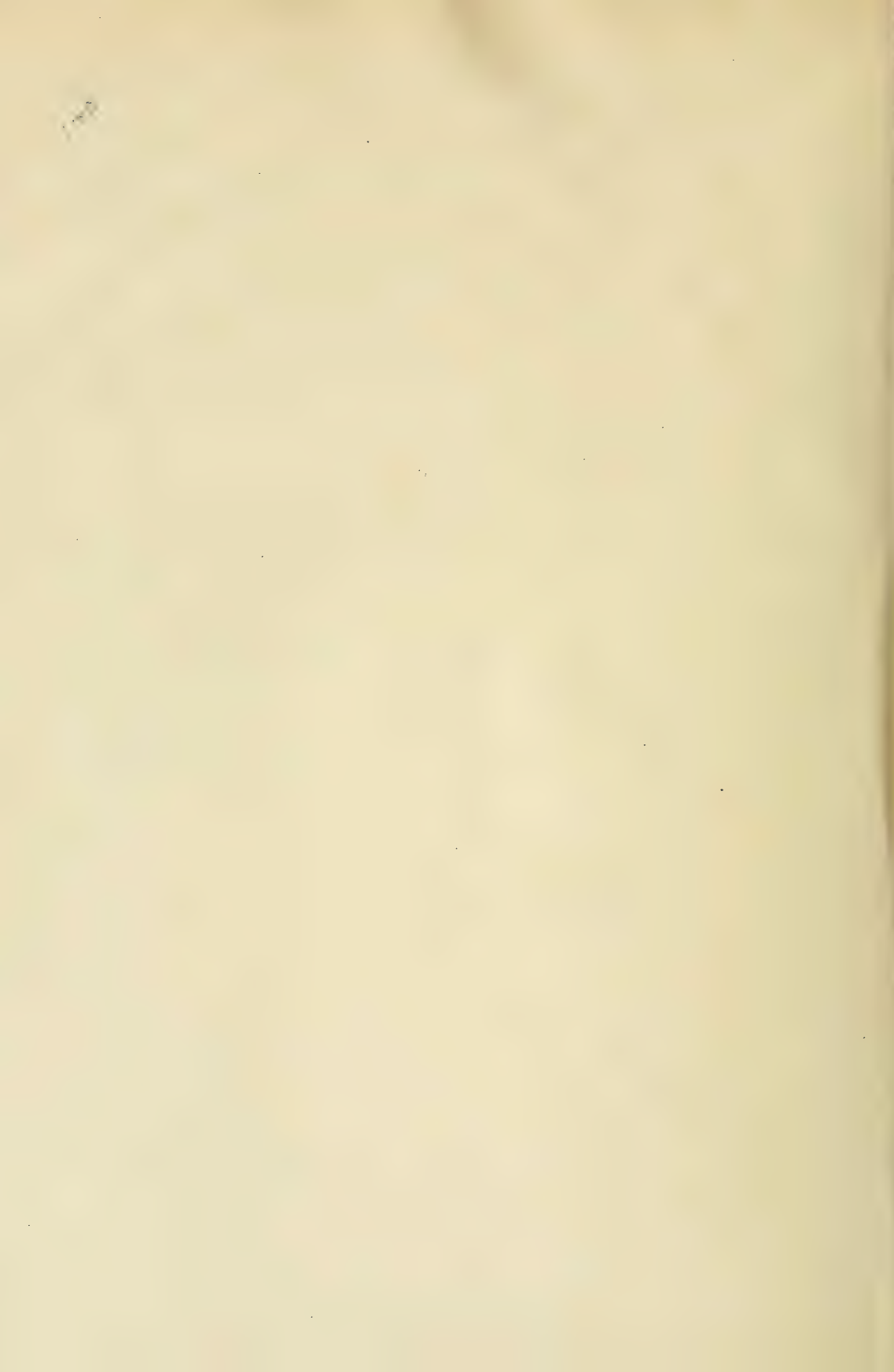
X. THE CONDENSATION OF TRYPTOPHANE AND OTHER INDOLE DERIVATIVES WITH CERTAIN ALDEHYDES.

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The oxidation of tryptophane by methods other than that adopted by Hopkins and Cole [1902] gives rise to coloured amorphous compounds. These observers, employing ferric chloride as the oxidising agent, isolated two substances, the one having the formula C_9H_7ON and afterwards identified by Ellinger [1906] as β-indolealdehyde; the other an interesting crystalline base, the analysis of which gave results in accordance with the formula $C_{12}H_{10}N_2$. This method of oxidation was used in the course of the present investigation with a view to the isolation and identification of the base $C_{12}H_{10}N_2$. Unfortunately, however, even though the conditions were varied as much as possible, none of this substance could be obtained. Ellinger, when preparing β-indolealdehyde by this method, had also failed to obtain Hopkins' base. In trying to ascertain the extent to which the ether used for extraction of indolealdehyde had been directly or indirectly responsible for the production of the base, Hopkins noticed that a crystalline derivative was formed when an aqueous solution of tryptophane was in contact with ether which had been heated locally with a glass rod. This compound however did not prove to be the much sought after base $C_{12}H_{10}N_2$, but a new substance of an acidic nature. At his suggestion the properties of the new substance, which may be conveniently spoken of as the " ether oxidation substance," were examined. This work led to an investigation of the direct action of formaldehyde, glyoxylic acid and glyoxal on tryptophane, and the action of trioxymethylene, in the presence of a condensing agent, on indole, skatole, indolepropionic acid, indoleacetic acid and tryptophane. It was found that the direct action of formaldehyde and glyoxylic acid on tryptophane led to the formation of *colourless* crystalline compounds; in these cases the *amino*-group of tryptophane was the reactive group (Section A). Glyoxal reacted directly with

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tryptophane in the presence of an oxidising agent to form a coloured derivative (Section B). For interaction with the *imino*-group of indole derivatives, the presence of a condensing agent is necessary, and the resulting products are all deeply *coloured* substances (Section C).

Section A.

I. *Preparation of the ether oxidation substance.*

(*a*) An aqueous solution of tryptophane was poured into a large wide-mouthed bottle fitted with a bung, and sufficient ether was added to form a layer a few mm. deep. In preliminary experiments the ether was heated locally by plunging a red-hot glass rod into the bottle and keeping it there a few minutes. Very pungent fumes were evolved, accompanied by a considerable rise in temperature. The process was repeated several times, and the bottle was well shaken and allowed to stand for a short time, when a crystalline deposit slowly formed. This somewhat tedious process was replaced by the following simple arrangement.

(*b*) The bung was pierced with four holes through which were passed a straight wide tube open at both ends, two electrodes, and an exit tube of narrow bore. The electrodes were connected inside the bottle by means of a platinum spiral placed just above the surface of the ether. The wide tube was arranged so as to have one end close to the platinum spiral. One end of the narrow tube was flush with the bung inside the bottle, the other end was connected with the vacuum pump and a slow current of air was drawn through the apparatus entering by the wide tube.

Outside the bottle the electrodes were connected with a battery sufficiently powerful to make the platinum spiral red-hot. The electric current was then switched off, and the current of air was regulated so that the heat developed during the incomplete combustion of the ether was sufficient to keep the platinum wire glowing. As soon as the layer of ether had disappeared, a further supply of ether was added, and the process repeated. The bottle was then disconnected, corked and left to stand at a temperature of 38° for several hours, during which time a crystalline deposit slowly formed. This substance proved to be the same as that prepared in (*a*).

(*c*) In another experiment a mixture of ether vapour and steam was passed over red-hot glass, and the ensuing vapours were passed through a solution of tryptophane ; under these conditions no ether oxidation substance

was formed. Thus it is evident that, to bring about the production of this substance, it is necessary for the tryptophane to be in contact with the reactive principle at the moment of its formation. The pungent fumes evolved during the slow incomplete combustion of ether were passed through a wash bottle containing water, and on examination were found to contain both hydrogen peroxide and formaldehyde.

(d) An attempt was made to prepare the substance by the action of formaldehyde and hydrogen peroxide on tryptophane. A crystalline derivative was obtained, but it did not prove to be the ether oxidation substance (post, p. 107).

In (a) and (b) the yield of crystalline product was 65 per cent. of the original tryptophane. The substance melts and decomposes at 324°, is colourless, crystalline, slightly soluble in water, alcohol and phenol, insoluble in cold and hot benzene, xylene, toluene, ethyl acetate, aniline, nitrobenzene, pyridine, amyl alcohol and ethylene dibromide. It is soluble in boiling glacial acetic acid; with concentrated hydrochloric acid it darkens considerably, and on evaporation in vacuo crystals separate out, which can be recrystallised from ether and alcohol and melt at 285°. Several analyses of this product were made, but failed to give concordant results. The substance can be boiled with dilute hydrochloric and sulphuric acids (5 %) without any decomposition taking place. It is precipitated by the mercury sulphate reagent, gives the glyoxylic but not the bromine tests, and does not darken with concentrated sulphuric acid. It is soluble in alkalis, and can be precipitated by means of acetic acid. The use of caustic alkalis is to be avoided, owing to the pigmentation which ensues. Its sodium salt cannot be recrystallised from water owing to hydrolysis.

The substance does not benzoylate easily, and does not form a naphthalene sulphochloride derivative. On reduction with phosphorus and hydriodic acid in a sealed tube at 280° it did not give products suitable for analysis. It is unattacked by alkaline permanganate. Fuming nitric acid reacts with the substance to yield a bright yellow nitro-compound which is soluble in water, giving a yellow solution capable of dyeing silk. On the addition of alkali to the yellow solution, the colour changes to deep red. Oxidation of the substance with chromium trioxide and glacial acetic acid, and with potassium bichromate, gave rise to brown amorphous substances. With concentrated sulphuric acid no colour reaction is produced unless a trace of formaldehyde be added. The purple colour produced under these conditions is identical with that produced by the addition of a trace of glyoxylic acid to the suspension of the substance in water.

A. The original crystalline compound prepared from solutions of recrystallised tryptophane by using methods (*a*) and (*b*) was filtered from the mother liquor, washed well with water, alcohol and ether, dried and analysed.

(1) Dried at the ordinary temperature *in vacuo*:

0·1600 g.; 0·3575 g. CO_2, 0·0880 g. H_2O. C=60·94, H=6·11 %.
0·1230 g.; 12 c.c. moist nitrogen at 15° C. and 768 mm. N=11·42 %.
$C_{24}H_{26}O_5N_4 . H_2O$ requires C=61·49, H=6·04, N=11·96 %.

(2) Heated to a temperature of 155°:

0·1533 g.; 0·3730 g. CO_2, 0·0870 g. H_2O. C=66·35, H=6·36 %.
0·1710 g.; 0·4180 g. CO_2, 0·0860 g. H_2O. C=66·66, H=5·63 %.
0·1050 g.; 11·6 c.c. moist nitrogen at 15° C. and 768 mm. N=13·00 %.
0·2080 g.; 21·2 c.c. moist nitrogen at 7° C. and 776 mm. N=12·90 %.
$C_{24}H_{24}O_4N_4$ requires C=66·66, H=5·61, N=12·96 %.

B. The compound was twice dissolved in ten per cent. sodium carbonate solution and precipitated with dilute acetic acid. The constant melting point 324° was obtained. The crystalline precipitate was washed well with water, alcohol and ether, dried at various temperatures and analysed.

(1) Dried *in vacuo* at the ordinary temperature:

0·1200 g.; 0·2683 g. CO_2; 0·0645 g. H_2O. C=61·10, H=6·02 %.
0·1080 g.; 11·1 c.c. moist nitrogen at 22° C. and 767 mm. N=11·95 %.
$C_{24}H_{26}O_5N_4 . H_2O$ requires C=61·49, H=6·04, N=11·96 %.

(2) Dried at temperatures between 110° and 150°:

0·1187 g.; 0·2804 g. CO_2; 0·0645 g. H_2O. C=64·42, H=6·04 %.
0·1014 g.; 0·2395 g. CO_2; 0·0549 g. H_2O. C=64·41, H=6·05 %.
0·1252 g.; 0·2950 g. CO_2; 0·0672 g. H_2O. C=64·3, H=6·01 %.
0·1914 g.; 20·9 c.c. moist nitrogen at 18° C. and 748 mm. N=12·51 %.
0·1912 g.; 20 c.c. moist nitrogen at 11·5° C. and 757·2 mm. N=12·43 %.
$C_{24}H_{26}O_5N_4$ requires C=64·00, H=5·84, N=12·45 %.

(3) Heated to temperatures between 150° and 200°: .

0·1037 g.; 0·2513 g. CO_2; 0·0545 g. H_2O. C=66·13, H=5·89 %.
$C_{24}H_{24}O_4N_4$ requires C=66·66, H=5·61, N=12·96 %.

It is thus evident that after solution of the ether oxidation substance in alkali and subsequent precipitation by acidification, the product precipitated has the same composition as when originally formed from the mother liquor.

It is of importance to note that the change in composition from $C_{24}H_{26}O_5N_4 . H_2O$ (*B* (1)) to $C_{24}H_{26}O_5N_4$ (*B* (2)) is of a reversible nature. That is to say, the ether oxidation substance previously heated to 140° will readily take up water again to give the monohydrate. But having effected the change

from $C_{24}H_{26}O_5N_4$ (B (2)) to $C_{24}H_{24}O_4N_4$ (B (3)), it is not possible to get the reverse reaction to take place. In other words, at temperatures above 150° the ether oxidation substance loses the elements of water as the result of some internal molecular rearrangement. The compound $C_{24}H_{24}O_4N_4$ does not undergo further change at temperatures from 200–220°, but it is decomposed at 225°, and carbon dioxide, ammonia and skatole are evolved.

II. *The condensation of formaldehyde and tryptophane.*

If a few cubic centimetres of formalin be added to a concentrated solution of pure tryptophane, and the reaction mixture be kept at a temperature of 38° for several hours, a crystalline derivative is deposited from the solution.

The compound melts and decomposes at 225–240°; it is slightly soluble in water and alcohol, insoluble in benzene and its homologues, amyl alcohol, chloroform, ethyl acetate and ethylene dibromide ; it is of a slightly yellow colour which becomes intensified if the substance be kept at a temperature of 150° for a short time, or if it be left exposed to the air at ordinary room temperature for some days. On being boiled with water, dilute acids and weak alkalis, it is readily hydrolysed, formaldehyde is liberated and the ether oxidation product is formed. With concentrated sulphuric acid the substance gives a purple colour, which is intensified if the suspension of the substance in water be heated and cooled before adding the concentrated acid.

The condensation product was formed as follows :

(*a*) By the interaction of tryptophane and samples of formaldehyde obtained (1) from Merck's, (2) from Kahlbaum's, and (3) from Schering's formalin, (4) by distilling Merck's trioxymethylene in a current of hydrogen or nitrogen and collecting the issuing gases in air-free water.

(*b*) The formaldehyde vapours evolved in (4) were passed into a solution of tryptophane from which all air had been dispelled by a current of hydrogen.

In each experiment a crystalline derivative melting at 235–240° was formed.

On account of the hydrolysis which takes place with water, acids and alkalis, the substance could not be purified by being dissolved in alkalis and re-precipitated by acid. For purposes of analysis it was filtered from the mother liquor, washed well with water, alcohol and ether, and dried at different temperatures. Analyses of samples prepared at different times and dried at the same temperature indicated constancy of composition.

(1) Of the substance dried *in vacuo*:

0·1208 g.; 0·2537 g. CO_2; 0·0700 g. H_2O.   C=57·30, H=6·49 %.
0·1135 g.; 0·2377 g. CO_2; 0·0658 g. H_2O.   C=57·10, H=6·56 %.
0·1340 g.; 0·2820 g. CO_2; 0·0763 g. H_2O.   C=57·33, H=6·38 %.
0·1175 g.; 10·7 c.c. moist nitrogen at 16° C. and 760 mm. N=10·81 %.
0·1512 g.; 14·4 c.c. moist nitrogen at 23° C. and 763·4 mm. N=10·95 %.
$C_{12}H_{12}O_2N_2 . 2H_2O$ requires C=57·14, H=6·35, N=11·11 %.

(2) Of the substance dried at temperatures between 110° and 130°:

0·1045 g.; 0·2355 g. CO_2; 0·0600 g. H_2O.   C=61·45, H=6·43 %.
0·1135 g.; 0·2540 g. CO_2; 0·0635 g. H_2O.   C=61·04, H=6·26 %.
0·1265 g.; 12·7 c.c. moist nitrogen at 25° C. and 765 mm. N=11·63 %.
$C_{12}H_{12}O_2N_2 . H_2O$ requires C=61·49, H=6·04, N=11·96 %.

(3) The substance was heated to 160° for ten minutes, but no analyses could be made owing to decomposition which began to take place; there was considerable carbonisation together with the elimination of skatole.

Analyses were made of the crystalline derivative formed by the hydrolysis of the formaldehyde product:

(1) *By water.* The derivative melted at 324°, and when mixed with some of the ether oxidation substance the melting point remained unchanged. The substance was dried at 110°.

0·1127 g.; 0·2678 g. CO_2; 0·0575 g. H_2O.   C=64·80, H=5·72 %.
0·1055 g.; 0·2475 g. CO_2; 0·0518 g. H_2O.   C=63·99, H=5·50 %.
0·1064 g.; 0·2520 g. CO_2; 0·0572 g. H_2O.   C=64·50, H=5·98 %.
0·1340 g.; 14·8 c.c. moist nitrogen at 19° C. and 768·4 mm. N=12·94 %.

(2) *By Na_2CO_3 solution.* The derivative melted at 324°.

(a) Of the substance dried *in vacuo*:

0·1028 g.; 0·2290 g. CO_2; 0·0565 g. H_2O.   C=60·80, H=6·16 %.

(b) Of the substance dried at 110°:

0·1000 g.; 0·2347 g. CO_2; 0·0525 g. H_2O.   C=64·09, H=5·83 %.
0·1135 g.; 12·3 c.c. moist nitrogen at 21° C. and 768 mm. N=12·74 %.

These results are in accordance with the assumption from the melting point that the product of hydrolysis is the ether oxidation substance ($C_{24}H_{26}O_5N_4 . H_2O$ and $C_{24}H_{26}O_5N_4$).

It is thus evident that, although the product formed by the condensation of formaldehyde with tryptophane is not the same as the ether oxidation substance, yet there is a close connexion between these two which can be demonstrated by the fact that on hydrolysis the former is readily converted into the latter substance.

III. *The action of formaldehyde and hydrogen peroxide on tryptophane* gave rise to a crystalline compound which proved to be the formaldehyde product II, and not the ether oxidation substance.

IV. *The condensation of glyoxylic acid with tryptophane.*

To an aqueous solution of tryptophane was added glyoxylic acid in the proportion of one molecule of tryptophane to one molecule of glyoxylic acid. A crystalline substance was deposited from the solution.

The substance was slightly soluble in water and alcohol, insoluble in ether, petroleum ether, benzene and its homologues, amyl alcohol, ethyl acetate and ethylene dibromide. It is soluble in alkalis, moderately soluble in dilute acids, readily in concentrated acids. It is precipitated by the mercury sulphate reagent. It gives the glyoxylic reaction and also the bromine colour tests characteristic of tryptophane. It does not give any colour reaction with concentrated sulphuric acid; on the addition of a trace of formaldehyde or of glyoxylic acid solution, the glyoxylic colour is formed: while on the addition of a trace of hydrogen peroxide a claret colour is obtained.

The substance, whether separated from the mother liquors and repeatedly washed with water, alcohol and ether, or recrystallised from hot water and from alcohol, melted at 324°, and when mixed with some of the ether oxidation substance the melting point was unaffected. That the derivative under discussion was not the ether oxidation substance was shown by the fact that when dissolved in sodium carbonate solution and the solution acidified with acetic acid, the *acid sodium salt* was precipitated and not the original condensation product. Analyses of the original acid (A), and of the acid sodium salt (B) were made and the results obtained were in accordance with the formulae $C_{13}H_{12}O_4N_2$ for the acid and $C_{13}H_{11}O_4N_2Na \cdot 4H_2O$ for the sodium salt.

A. Analyses of the original product (m.p. 324°).

(1) Of the substance dried in vacuum desiccator:

0·1238 g.; 0·2693 g. CO_2; 0·0542 g. H_2O. C=59·34, H=4·89 %.
0·1858 g.; 18 c.c. moist nitrogen at 21°C. and 777·3 mm. N=11·21 %.

(2) Dried at 150°:

0·1008 g.; 0·2193 g. CO_2; 0·0433 g. H_2O. C=59·35, H=4·82 %.
0·2820 g.; 26·2 c.c. moist nitrogen at 20°C. and 757·2 mm. N=10·70 %.

(3) Heated to 200°:

0·1055 g.; 0·2287 g. CO_2; 0·0465 g. H_2O. C=59·12, H=4·99.
(1), (2) and (3). $C_{13}H_{12}O_4N_2$ requires C=59·97, H=4·66, N=10·77 %.

(4) Heated to temperatures between 205° and 220° for ten minutes, carbon dioxide being evolved.

> 0·1184 g.; 0·2875 g. CO_2; 0·0575 g. H_2O. C=66·23, H=5·44 %.
> 0·1525 g.; 16·8 c.c. moist nitrogen at 14·5° C. and 755·3 mm. N=12·94 %.
> $C_{21}H_{24}O_4N_4$ (ether oxidation substance) requires C=66·66, H=5·61, N=12·96 %.

It is evident from the above analyses that at temperatures between 15° and 200° the glyoxylic condensation product has a constant composition; at 220° it is converted, with loss of carbon dioxide, into the ether oxidation substance.

(5) If the glyoxylic compound be heated for a short time at a temperature of 225–230° it decomposes, yielding carbon dioxide, ammonia and skatole (cf. I, p. 105).

B. The acid sodium salt (see above) was twice recrystallised from water and analysed.

> 0·3735 g.; 26·1 c.c. moist nitrogen at 20·8° C. and 757·3 mm. N=8·08 %.
> 0·1289 g.; 9 c.c. moist nitrogen at 21·5° C. and 761·9 mm. N=8·02 %.
> 0·2048 g.; ·0406 g. Na_2SO_4. Na=6·42 %.
> 0·3700 g.; 0·0562 g. Na_2CO_3 and 0·0762 g. Na_2SO_4. Na=6·59 and 6·67 %.
> 0·8122 g.; 0·1542 g. Na_2SO_4. Na=6·15 %.

From these determinations the molecular weight of the substance = 355.

> 0·6074 g.; 0·4890 g. anhydrous residue,
> ∴ in the acid salt there are 3·84 mols. water.
> 0·1033 g.; 0·0528 g. H_2O; 0·1612 g. CO_2.
> Putting in the correction for the Na_2CO_3 remaining in the boat (0·0150 g.) } C=44·20, H=5·72 %.
> $C_{13}H_{11}O_4N_2Na . 4H_2O$ requires N=7·91, C=44·07 %.
> H=5·37, Na=6·50 %.
> M.W. 354·3.

From these results it is obvious that the glyoxylic condensation product and the ether oxidation substance are two distinct compounds, in spite of the fact that the compounds when taken separately and also when mixed together have the same melting point, viz. 324°. It is obvious that the glyoxylic compound readily changes to the ether oxidation substance on being heated to a temperature of 205°. (The ether oxidation substance is not affected under the same conditions.) At 225° both are decomposed into carbon dioxide, ammonia, skatole, etc.

There is thus an interesting connexion between the formaldehyde compound, the glyoxylic condensation product, and the ether oxidation substance. The last-named substance can be produced from the formaldehyde product by hydrolysis, and from the glyoxylic condensation product by the application of heat.

It is probable from the following reasons that these compounds described under I, II and IV are formed by the simple process of condensation between the $-NH_2$ group of tryptophane and the $-CHO$ group of the reacting aldehydes:

1. The ease with which the reactions take place (the presence of a condensing agent is unessential).

2. The failure to produce naphthalene sulphochloride derivatives points to the substitution of the hydrogen of the amino-groups.

3. The fact that the compounds so obtained are colourless points to the condensation having taken place as a result of the activity of the amino- and not the imino-group (*post*, p. 112).

4. Corresponding compounds could not be obtained with indole, skatole, indoleacetic and indolepropionic acids, i.e. with indole derivatives in which there is no *amino*-group.

For reaction to take place with these compounds, the presence of a condensing agent is necessary, and the resulting products are intensely coloured (*post*, p. 112).

Analyses of the three compounds I, II, IV are in accordance with the following formulae :

(a)	The formaldehyde product	...	...		$C_{12}H_{12}O_2N_2.2H_2O.$
(b)	The glyoxylic product...	...	...		$C_{13}H_{12}O_4N_2.$
	,, ,, ,, above 205°		...		$C_{24}H_{21}O_4N_4.$
(c)	The ether oxidation substance		...		$C_{24}H_{26}O_5N_4.$
	,, ,, ,, ,, above 150°				$C_{24}H_{24}O_4N_4.$

The mechanism involved in the production of these three substances may be briefly described as follows :

I. Since the *ether oxidation product* is in no way an oxidation product of tryptophane, but is closely connected with the formaldehyde condensation product, it is suggested that in the formation of the ether oxidation substance nascent formaldehyde is the active principle (p. 102, I c).

$$R.CH_2.CH(NH_2).COOH + 2HCHO \text{ (nascent)} \rightarrow R.CH_2.\overset{\overset{\displaystyle COOH}{|}}{CH}.NH.CH_2.O.CH_2.NH.\overset{\overset{\displaystyle HOOC}{|}}{CH}.CH_2R$$

(where R represents the indole nucleus C_8H_6N).
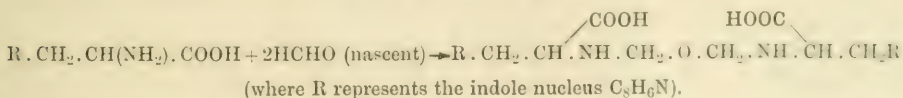
On heating this substance to a temperature of 150–200°, molecular water and not water of crystallisation is lost. After such a process the composition of the substance corresponds to the formula $C_{24}H_{24}O_4N_4$, and can be represented graphically as

$$\overset{\displaystyle COOH}{\underset{\displaystyle R.CH_2}{}}\!\!>CH.NH.CH=CH.NH.CH<\!\!\overset{\displaystyle COOH}{\underset{\displaystyle CH_2.R}{}}$$
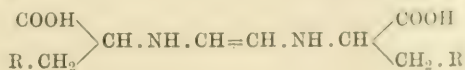
II.   *The formaldehyde condensation product* is formed as follows:

$$R.CH_2.CH(NH_2).COOH + HCHO = R.CH_2.CH \Big\langle {}^{COOH}_{N:CH_2} + H_2O$$

III.   The *glyoxylic condensation product* is produced in a similar manner:

$$R.CH_2.CH(NH_2).COOH + COOH.CHO = R.CH_2.CH \Big\langle {}^{COOH}_{N:CH.COOH} + H_2O$$
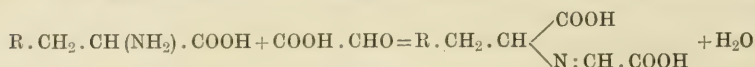
It has been shown that the ether oxidation substance is readily formed (*a*) from the formaldehyde condensation product by hydrolysis, (*b*) from the glyoxylic condensation product by the application of heat.   For the former process (*a*) there are two possibilities to be considered, viz.:

(1)   That during hydrolysis the molecules are linked together by means of the addition of the elements of water, thus:

$$2R.CH_2.CH \Big\langle {}^{COOH}_{N:CH_2} + H_2O = R.CH_2.CH.NH.CH_2.O.CH_2.NH.CH.CH_2.R$$
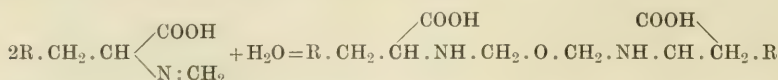
(2)   That during the hydrolysis tryptophane and nascent formaldehyde are re-formed, and that these react as in the ether oxidation process to form the substance $C_{24}H_{26}O_5N_4$.

(*b*)   In the case of the production of the substance $C_{24}H_{24}O_4N_2$ from the glyoxylic product by the application of heat, it is evident that two molecules of the latter each lose a molecule of carbon dioxide, and at the temperature at which the reaction takes place condensation of these two molecules occurs, accompanied by a rearrangement of the bonds into the more stable configuration of the ether oxidation substance.

$$2R.CH_2.CH(COOH).N:CH.COOH$$
$$= R.CH_2.CH(COOH).NH.CH:CH.NH.CH(COOH).CH_2.R + 2CO_2.$$

A study of the properties of these three compounds throws some light on the mechanism of the Adamkiewicz reaction: a discussion of this point will be dealt with in the following paper [Homer, 1913, p. 117, Table I].

Section B.

The condensation of glyoxal with tryptophane.

If an aqueous solution of tryptophane heated to a temperature of $38°$ be treated with the calculated quantity of glyoxal in the presence of hydrogen peroxide, a crystalline chocolate-coloured deposit slowly forms.

This coloured substance is of an acidic nature and is slightly soluble in water and in alcohol.   It dissolves in alkalis, and is moderately soluble in

dilute acids, and with the exception of acetic acid readily soluble in concentrated acids. It is precipitated by the mercury sulphate reagent. With concentrated sulphuric acid it gives a brown colour which in the presence of hydrogen peroxide changes to claret. In the presence of a trace of formaldehyde, a brown colour is produced which is changed to the colour of bromine on the addition of an oxidising agent.

Analyses of the substance gave the following results:

(1)   Of the substance recrystallised from water and

   (a)   Dried at 110°:

$0 \cdot 1030$ g.; $0 \cdot 2325$ g. CO_2; $0 \cdot 0463$ g. H_2O. $C = 61 \cdot 60$, $H = 5 \cdot 04$ %.
$0 \cdot 1223$ g.; $0 \cdot 2780$ g. CO_2; $0 \cdot 0560$ g. H_2O. $C = 61 \cdot 99$, $H = 5 \cdot 13$ %.
$0 \cdot 1025$ g.; $0 \cdot 2346$ g. CO_2; $0 \cdot 0185$ g. H_2O. $C = 62 \cdot 40$, $H = 5 \cdot 25$ %.
$0 \cdot 2268$ g.; $21 \cdot 6$ c.c. moist nitrogen at $24°$ C. and $767 \cdot 5$ mm. $N = 10 \cdot 9$ %.
$C_{26}H_{22}O_6N_4 . H_2O$ requires $C = 61 \cdot 90$, $H = 4 \cdot 81$, $N = 11 \cdot 1$ %.

   (b)   Heated to 130°:

$0 \cdot 1006$ g.; $0 \cdot 2360$ g. CO_2; $0 \cdot 0412$ g. H_2O. $C = 64 \cdot 00$, $H = 4 \cdot 62$ %.
$0 \cdot 1080$ g.; $10 \cdot 7$ c.c. moist nitrogen at $21°$ C. and 768 mm. $N = 11 \cdot 51$ %.

(2)   After solution in Na_2CO_3 and precipitation by acetic acid:

$0 \cdot 1430$ g.; $0 \cdot 0565$ g. H_2O; $0 \cdot 3403$ g. CO_2. $C = 64 \cdot 90$, $H = 4 \cdot 43$ %.
$0 \cdot 1060$ g.; $10 \cdot 2$ c.c. moist nitrogen at $20°$ C. and 769 mm. $N = 11 \cdot 24$ %.

(3)   Crystallised from alcohol and heated to 110°:

$0 \cdot 1017$ g.; $\cdot 0441$ g. H_2O; $\cdot 2390$ g. CO_2. $C = 64 \cdot 15$, $H = 4 \cdot 86$ %.
$0 \cdot 1290$ g.; $12 \cdot 5$ c.c. moist nitrogen at $19°$ C. and $750 \cdot 5$ mm. $N = 11 \cdot 1$ %.

(4)   Crystallised from pyridine:

$0 \cdot 1060$ g.; $\cdot 0441$ g. H_2O; $0 \cdot 2505$ g. CO_2. $C = 64 \cdot 45$, $H = 4 \cdot 63$ %.
Ib. 2, 3, 4:—$C_{26}H_{22}O_6N_4$ requires $C = 64 \cdot 2$, $H = 4 \cdot 53$, $N = 11 \cdot 5$ %.

(5)   Heated to 160°:

$0 \cdot 1006$ g.; $0 \cdot 0410$ g. H_2O; $0 \cdot 2459$ g. CO_2. $C = 66 \cdot 66$, $H = 4 \cdot 53$ %.
$0 \cdot 1150$ g.; $0 \cdot 0460$ g. H_2O; $0 \cdot 2830$ g. CO_2. $C = 67 \cdot 07$, $H = 4 \cdot 46$ %.
$0 \cdot 1890$ g.; $19 \cdot 6$ c.c. moist nitrogen at $16°$ C. and $749 \cdot 6$ mm. $N = 12 \cdot 01$ %.
$C_{26}H_{20}O_5N_4$ requires $C = 66 \cdot 66$, $H = 4 \cdot 32$, $N = 11 \cdot 97$ %.

Taking into consideration the necessity for the presence of an oxidising agent, and also the fact that the substance produced is intensely coloured, it is highly probable that in this reaction, besides the simple aldehyde condensation occurring in the reactions described in Section A, there has also been elimination of hydrogen accompanied by complex ring formation.

Possible equation:

$$2C_{11}H_{12}O_2N_2 + 2 (CHO)_2 + O = C_{26}H_{22}O_6N_4 . H_2O + 2H_2O.$$
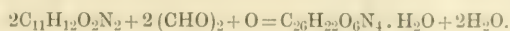
Any attempt to ascertain the constitution of this substance has been postponed for the present.

<center>SECTION C.</center>

THE PRODUCTS FORMED BY THE CONDENSATION OF ALDEHYDES WITH THE IMINO-GROUP OF INDOLE DERIVATIVES.

I. *The action of formaldehyde on indole derivatives in presence of a condensing agent.*

In connexion with the work on the condensation of formaldehyde with tryptophane, an attempt was made to prepare corresponding derivatives with indole, skatole, indolepropionic and indoleacetic acids, but without success. However, it is well known that under suitable conditions, in the presence of concentrated sulphuric acid, a solution of formaldehyde reacts with the above-mentioned compounds to yield coloured substances. Concentrated sulphuric acid being known to be both an oxidising and a condensing agent, the question arose as to whether the colour effect was due to a process of simple condensation between formaldehyde and the above-mentioned substances, which have an imino-group in common, or was in any way dependent upon the oxidising power of the acid.

In order to test this suggestion, it was thought advisable to avoid the use of sulphuric acid and attempts were made to form condensation products of indole derivatives with trioxymethylene, using zinc chloride and alcohol saturated with hydrochloric acid gas as the condensing agents (with these condensing agents an aqueous solution of formaldehyde could not be used).

II. *Derivatives formed by the interaction of trioxymethylene and indole derivatives, using zinc chloride as the condensing agent.*

Indole, and indoleacetic acid reacted readily at temperatures below 100° to produce purple-coloured products. Skatole and indolepropionic acid under the same conditions gave brown products. Owing to scarcity of material, the products from indoleacetic and indolepropionic acids could not be analysed. The indole and skatole derivatives were investigated.

Tryptophane treated in the same way did not react until heated to a much higher temperature (160°); the resulting product was of a *deep brown* colour. The reaction was accompanied by a certain amount of decomposition of tryptophane. As it was impossible to free the product of the reaction from the results of carbonisation, no further investigation of this substance has been made.

Preparation of the trioxymethylene derivatives of skatole and indole. One part by weight of indole or skatole was mixed with the same weight of trioxymethylene and ground together with one part by weight of freshly powdered and freshly ignited zinc chloride. The process of grinding the reaction mixture gave rise in the former case to the formation of a purple colour. The reaction mixture was heated on a water-bath to a temperature of 80° for half an hour, and then transferred to an oil bath and the temperature raised to 150° for a few minutes. On cooling, the coloured mixture was treated with hydrochloric acid solution (one part concentrated acid to one part water) and allowed to stand for some hours. The residue was in each case repeatedly boiled with dilute hydrochloric acid, and then with water until free from formaldehyde and hydrochloric acid. The coloured substance was finally washed with water, alcohol and ether, dried and analysed.

It was impossible to purify the products further on account of their insolubility in all liquids other than concentrated sulphuric acid. Constancy of composition was denoted by analyses of samples prepared at different times and under slightly different conditions as regards the proportion of zinc chloride used. It is interesting to note that the indole compound is of a purplish colour, the skatole compound is brown. They are both insoluble in ordinary solvents, in alkalis and in acids with the exception of concentrated sulphuric acid, in which they are appreciably soluble, and concentrated hydrochloric acid in which they are slightly soluble. In concentrated sulphuric acid the indole derivative gives a reddish violet colour which on dilution becomes bluish and finally disappears owing to the decreasing solubility of the substance as the acid becomes more dilute. The skatole compound under the same conditions gives rise to a brown colour.

(1) Analyses of the indole derivative prepared on different occasions were made [1].

A. 0·1026 g.; 0·0475 g. H_2O; 0·2702 g. CO_2. C = 71·85, H = 5·20 °/₀.
B. 0·1057 g.; 0·0518 g. H_2O; 0·2783 g. CO_2. C = 71·80, H = 5·49 °/₀.

A. 0·1325 g.; 9·4 c.c. moist nitrogen at 14° C. and 753·5 mm. N = 8·32 °/₀.
   0·1500 g.; 11·0 c.c. moist nitrogen at 15·5° C. and 747·3 mm. N = 8·34 °/₀.
B. 0·2074 g.; 14·2 c.c. moist nitrogen at 13° C. and 755·3 mm. N = 8·07 °/₀.

Analysis of the indole derivative after being heated to 205° for fifteen minutes gave the following result:

0·1045 g.; 0·0502 g. H_2O; 0·2750 g. CO_2. C = 71·78, H = 5·38 °/₀.

Hence the compound is stable to heat at temperatures not exceeding 205°.

$C_{21}H_{26}O_3N_2$ requires C = 72·41, H = 5·74, N = 8·04 °/₀.

[1] These compounds are extremely difficult to burn and this probably accounts for the slightly low values obtained.

(2) Analyses of the skatole derivative gave the following results[1]:

0·1161 g. ; 0·0658 g. H_2O ; 0·3120 g. CO_2.   C = 73·30, H = 6·35 %.
0·1083 g. ; 0·0575 g. H_2O ; 0·2910 g. CO_2.   C = 73·30, H = 5·94 %.
0·1685 g. ; 9·8 c.c. moist nitrogen at 13·5° C. and 758·5 mm.   N = 7·13 %.
0·1820 g. ; 10·8 c.c. moist nitrogen at 11·5° C. and 765 mm.   N = 7·15 %.
$C_{23}H_{21}O_3N_2$ requires C = 73·40, H = 6·38, N = 7·42 %.

The condensations may therefore be represented by the equations:

$$2C_8H_7N + 5HCHO = C_{21}H_{20}O_3N_2 + 2H_2O$$
$$2C_9H_9N + 5HCHO = C_{23}H_{21}O_3N_2 + 2H_2O.$$
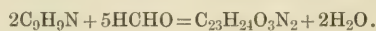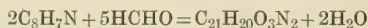
It is thus obvious that in the presence of a condensing agent trioxymethylene reacts with the imino-group of the indole nucleus to form coloured bodies.

III. *Derivatives formed by the interaction of trioxymethylene and indole derivatives, using a solution of hydrochloric acid gas in alcohol as the condensing agent.*

A description of the condensation products with indole, skatole and tryptophane formed under these conditions will form the subject-matter of a future paper. It may be stated here that they are intensely coloured substances, and that the indole derivative is the only one of the three which will give a purple colour with concentrated sulphuric acid.

The derivatives formed by the action of trioxymethylene on indole derivatives in the presence of a condensing agent, whether it be concentrated sulphuric acid, or zinc chloride, or an alcoholic solution of hydrochloric acid gas, are all characterised by their insolubility in solvents other than concentrated mineral acids.

A discussion as to the significance of activity of the *imino*-group of indole derivatives in the production of the specific colour reaction known as the Adamkiewicz reaction, will be found in the paper following this [Homer, 1913, p. 116].

During the course of the work under consideration it has been noticed that tryptophane itself under certain conditions of oxidation gives rise in the process of time to the formation of pigments having definite absorption bands. The properties both chemical and physical of these pigments, and of those formed from normal breakdown products of tryptophane, are being investigated, and will form the subject of a future paper. At this

[1] These compounds are extremely difficult to burn and this probably accounts for the slightly low values obtained.

point it may be observed that in nearly every case these substances are themselves brown in colour, and their solutions in organic solvents are also brown, but the presence of acid changes the colour from brown to purple.

Summary.

1. Tryptophane by virtue of its $-NH_2$ group reacts directly with nascent formaldehyde, formaldehyde and glyoxylic acid to form colourless crystalline compounds.

2. Glyoxal will react with tryptophane in the presence of an oxidising agent to form an intensely coloured compound.

3. Indole derivatives, by virtue of the $-NH$ group in the nucleus, will react with formaldehyde and trioxymethylene *in the presence of a condensing agent* to form substances of intense colour and marked insolubility in ordinary solvents other than concentrated mineral acids.

REFERENCES.

Ellinger (1906), *Ber.* **39**, 2, 2515.
Homer (1913) *Biochem. J.* **7**. 116.
Hopkins and Cole (1902), *J. Physiol.* **29**, 464.

XI. ON THE COLOUR REACTIONS OF CERTAIN INDOLE DERIVATIVES, AND THEIR SIGNIFICANCE WITH REGARD TO THE GLYOXYLIC REACTION.

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In the preceding paper [Homer, 1913] the preparation and properties of several new indole derivatives have been described in detail.

The condensation products formed between tryptophane and nascent formaldehyde, formaldehyde and glyoxylic acid respectively in the absence of a condensing agent, are colourless crystalline compounds. The reaction takes place between the $-CHO$ group of the aldehyde and the $-NH_2$ group of tryptophane.

In the presence of condensing agents, such as zinc chloride or an alcoholic solution of hydrochloric acid gas, indole derivatives and pyrrole, by virtue of the $-NH$ group in the nucleus, combine with trioxymethylene to form coloured substances; in the presence of concentrated sulphuric acid a solution of formaldehyde reacts with indole and pyrrole derivatives to give colour reactions.

In the case of the condensation of glyoxal with tryptophane, the presence of an oxidising agent is necessary, and the resulting product, which is coloured, no longer gives the glyoxylic reaction.

The results of an investigation of the colour reactions of the above-mentioned substances have been embodied in the following tables, and afford special interest with regard to the mechanism of (*a*) the Adamkiewicz reaction for the detection of proteins and (*b*) Hopkins and Cole's glyoxylic reaction.

Section I.

(In the following reactions, air-free water has been used in every case.)

TABLE I. *Colour reactions produced by the action of pure concentrated sulphuric acid on the formaldehyde, and glyoxylic acid condensation products of tryptophane, on the ether oxidation substance and on tryptophane.*

Action of concentrated sulphuric acid	Tryptophane	Formaldehyde product	Glyoxylic condensation product	Ether oxidation substance
A. On the solid :				
i. In the cold	None	Purple[1]	Yellow	None
ii. On warming	Slight darkening. Finally charring	Colour intensified. Finally charring	Colour intensified. Finally charring	Slight purple tint
B. On the solid suspended in water :				
i. Directly treated with the acid	None	Purple	Yellow	None
ii. Heated to 90°, cooled and then treated with the acid	None	Purple colour intensified	Yellow	None
iii. In the presence of one drop of a 1/200 solution of formalin in water	Brown	Purple colour intensified	Purple	Purple
iv. In the presence of a trace of glyoxylic acid	Purple	Purple	Purple	Purple
v. In the presence of a trace of ferric chloride	Orange red	Very intense purple	Brown	Green
vi. In the presence of a trace of hydrogen peroxide	Orange red	Brown	Claret	Green

[1] The term "purple" has been used for colours that are red with a tinge of blue in them, or vice versâ.

TABLE II. *The colour reactions produced by the action of concentrated sulphuric acid on pyrrole and indole derivatives in presence of glyoxal.*

Action of concentrated sulphuric acid	Pyrrole	Indole	Skatole	Indole-propionic acid	Tryptophane
In the presence of :					
i. A trace of glyoxal ...	Reddish brown	Indian red	Burnt Sienna	Reddish brown	Claret
to which has been added a trace of :					
(a) Ferric chloride ...	Greenish tint	Greenish tint	Reddish brown	Deep orange which rapidly changes to purple madder tint	Claret colour intensified
(b) Hydrogen peroxide...					
ii. An infinitesimal trace of glyoxal	Yellow brown	Very delicate shade of Indian red	Reddish brown	Yellowish brown with a tinge of red	Russet
to which has been added a trace of :					
(a) Ferric chloride ...	Intense blue changing to bluish purple	Green	Reddish brown	Orange red which rapidly changes to a purple madder	Russet colour intensified
(b) Hydrogen peroxide...	Green	Green	Magenta		

TABLE III. *The colour reactions produced by the action of pure concentrated sulphuric acid on pyrrole and various indole derivatives in the presence of* (A) *formaldehyde, and* (B) *glyoxylic acid.*

Action of concentrated sulphuric acid	Pyrrole	Indole	Skatole	Indole-propio-nic acid	Trypto-phane	Glyoxylic conden-sation product	Ether oxida-tion sub-stance
A. i. In the presence of 1 drop of a solution of 1 c.c. of formalin in 10 c.c. of water	Red brown	Purple	Brown	Brown	Brown	Purple	Purple
ii. In the presence of 1 drop of a 1/200 solution of formalin in water	Red brown	Purple	Brown	Brown	Brown	Purple	Purple
to which has been added a trace of an oxidising agent	Green	Purple	Purple	Purple	Purple	Purple	Purple
iii. In the presence of 1 drop of a 1/2000 solution of formalin in water	Yellow-ish brown	Purple	Purple	Purple	Purple	Purple	Purple
iv. In the presence of 1 drop of a solution of formaldehyde made by dissolving 1 part of trioxymethylene in 1000 parts boiling water	Red brown	Purple	Brown	Brown	Brown	Purple	Purple
v. In presence of 1 drop of the solution used in iv, diluted ten times	Yellow-ish brown	Purple	Purple	Purple	Purple	Purple	Purple
B. In the presence of glyoxylic acid	Red brown	Purple	Purple	Purple	Purple	Purple	Purple

TABLE IV. *The colour reactions produced by the direct action of pure concentrated sulphuric acid on the trioxymethylene condensation derivatives of indole, skatole and tryptophane.*

(a) Formed in the presence of zinc chloride as condensing agent.

(b) Formed in the presence of an alcoholic solution of hydrochloric acid gas.

Action of concentrated sulphuric acid	Indole derivative	Skatole derivative	Tryptophane derivative
(a) On the solid	Purple	Brown	Brown
(b) On the solid	Purple	Brown	Brown

Tables V *a* and V *b* illustrate the masking effect due to the presence of trioxymethylene.

TABLE V *a.* *Colour reactions produced by the action of pure concentrated sulphuric acid on aqueous solutions or suspensions of indole derivatives.*

Action of pure concentrated sulphuric acid	Indole	Skatole	Indole-propionic acid	Trypto-phane	Glyoxylic condensation product and ether oxidation substance
i. In the presence of 1 drop of a 1/200 solution of formalin in water	Purple	Brown	Brown	Brown	Purple
to which has been added an oxidising agent	Purple	Purple	Purple	Purple	Purple
ii. In the presence of a trace of trioxymethylene	Purple	Brown	Brown	Brown	Purple
iii. In the presence of 1 drop of a 1/200 solution of formalin in water, an oxidising agent, and a trace of trioxymethylene, cf. i (above)	Purple	Brown	Momentary appearance of purple colour, immediately masked by brown colour	Brown	Purple

TABLE V *b.* *Colour reactions produced by the action of concentrated sulphuric acid, in which has been dissolved a trace of trioxymethylene, on aqueous solutions or suspensions of indole derivatives.*

Action of concentrated sulphuric acid, containing a trace of trioxymethylene	Indole	Skatole	Indole-propionic acid	Trypto-phane	Glyoxylic condensation product and ether oxidation substance
i. Directly on the aqueous solution or suspension	Purple	Brown	Brown	Brown	Purple
ii. In the presence of an oxidising agent	Purple	Brown	Momentary appearance of purple, very soon masked by predominating brown	Brown	Purple
iii. In the presence of an oxidising agent and 1 drop of a 1/200 solution of formalin in water	Purple	Brown	Momentary appearance of purple, very soon masked by predominating brown	Brown	Purple

Adamkiewicz [1874] demonstrated that a violet colour is produced by the action of concentrated sulphuric acid on an acetic acid solution of protein. Hopkins and Cole [1900; 1902] showed that the protein reacts by virtue of its tryptophane groupings, and that *pure* acetic acid does not give the colour test. As a result of their investigations they were led to the conclusion that the reactive impurity in the acetic acid was glyoxylic acid.

Therefore, in the Adamkiewicz reaction, Hopkins and Cole regarded

glyoxylic acid as the substance essential to the formation of the characteristic violet colour. Rosenheim [1906] however, points out that the colour produced by the action of formaldehyde on proteins in presence of sulphuric acid and oxidising agents [Voisenet 1905] is identical with that produced in the Adamkiewicz reaction. He criticises Hopkins and Cole's view as to the importance of glyoxylic acid *per se*; he suggests that even with glyoxylic acid an oxidising agent is essential, and that although the latter may not have been added, yet the glyoxylic acid itself, however carefully prepared, is always contaminated with sufficient hydrogen peroxide for the reaction to take place. On the other hand Dakin [1909], noticing that the colours produced by the action of glyoxylic acid and formaldehyde are identical, suggests that in the case of formaldehyde, under the influence of acids, an aldol condensation and subsequent oxidation to glyoxylic acid take place.

From Table I it is obvious that formaldehyde *per se* plays an important part in the colour reaction, since of the three condensation products, the only one which will give the colour without the addition of glyoxylic acid or formaldehyde, is the formaldehyde condensation product. Further it has been shown that this product is readily hydrolysed with liberation of formaldehyde; at the line of junction of concentrated acid and water such hydrolysis would take place. A proof of this assumption lies in the observation that after boiling this product with water and so ensuring hydrolysis, the colour reaction is still more marked (Table I, B ii).

Were the essential factor glyoxylic acid *per se*, the glyoxylic condensation product ought to give the colour reaction with sulphuric acid, but it will not do so unless formaldehyde or more glyoxylic acid be added. Now glyoxylic acid itself is decomposed by sulphuric acid with the formation of carbon dioxide and formaldehyde[1]. It therefore seems reasonable to assume that glyoxylic acid is able to take part in the colour reaction by virtue of its decomposition into formaldehyde, and not, as Rosenheim suggests, because of contamination with hydrogen peroxide. The fact that the colour reaction is not given by glyoxal (Table II) and glycollic aldehyde, neither of which gives formaldehyde on treatment with acid, is in support of this view.

On the other hand, if formaldehyde be the essential factor, then some explanation must be offered to account for the generally acknowledged uncertainty of the colour reaction when that reagent is used, whereas with glyoxylic acid there is no difficulty experienced in carrying out the test for indole derivatives.

[1] A small quantity of glyoxylic acid was heated with 5 c.c. of water and 5 c.c. of concentrated sulphuric acid. The gases evolved were found to contain formaldehyde and carbon dioxide.

According to Hopkins and Cole, Dakin, and Rosenheim, the presence of an oxidising agent is necessary for the Adamkiewicz reaction to be carried out with formaldehyde.

In the present investigation samples of pure sulphuric acid (Kahlbaum and others) were specially procured and carefully selected so that they did not induce a purple colour with tryptophane and a 1/500 solution of formalin in air-free water. The addition of a trace of ferric chloride solution to the reacting liquids induced the purple colour to appear. Samples of sulphuric acid contaminated with a trace of an oxidising agent caused the appearance of the purple colour with the use of a 1/100, and in some cases a 1/10 solution of formalin in water.

A study of Table III shows that, using a 1/200 solution of formalin in water, without the addition of an oxidising agent, indole, indolealdehyde, indoleacetic acid, and the condensation products of tryptophane gave a purple colour, skatole, tryptophane and indolepropionic acid gave brown colourations. The addition of a trace of an oxidising agent to the latter was necessary for the production of the purple colour. In this connexion the following observations have been made:

1. In applying the formaldehyde test to tryptophane and skatole, if previous to the addition of the above-mentioned formaldehyde solution a trace of indolealdehyde be added, then the presence of the concentrated sulphuric acid will induce the formation of the characteristic purple colour, even though no oxidising agent has been added. This experiment shows that for the production of the purple colour the indole derivative and not the formaldehyde is attacked by the oxidising agent.

2. If to an aqueous solution of tryptophane one or two drops of formalin be added, the liquid boiled, cooled, diluted and treated with concentrated sulphuric acid, then the characteristic purple is produced, but is soon masked by the brown colouration usually noticed.

In this experiment the presence of one of the condensation products of tryptophane which gives a purple colour with formaldehyde has been assured.

In order to ascertain whether this purple colour reaction was due to an aldol condensation and oxidation of formaldehyde to glyoxylic acid [Dakin, 1909], the formalin itself was boiled, cooled, diluted to 1/200 and added to a cold aqueous solution of tryptophane. There was no production of a purple colour.

It will be seen from Table III, A iii and v that if the formaldehyde be diluted to the order of 1 part to 2000 or 20,000 parts of water, then the

purple colour is formed with skatole, tryptophane and indolepropionic acid without the addition of an oxidising agent. This result was obtained with Kahlbaum's and Merck's formalin, and also with a solution of formaldehyde made by dissolving trioxymethylene in boiling water which had been previously boiled for some time to expel air. The substances to be tested were dissolved in air-free water. Even with such large dilution, there is in the case of tryptophane a certain amount of brown colouration produced, but not sufficient to mask the purple colour. As the concentration of the formalin is increased, so the brown colour preponderates and masks the purple.

These experiments, taken in conjunction with the fact that the formalde-hyde condensation product is the only one which gives the Adamkiewicz reaction without the addition of glyoxylic acid or formaldehyde, seem to be conclusive as to the importance of formaldehyde *per se* as a factor in this reaction. But at the same time, in performing the Adamkiewicz colour reaction the fact that concentrated sulphuric acid causes the condensation of formaldehyde to trioxymethylene must be taken into consideration. In the previous paper it has been shown that trioxymethylene, in the presence of a condensing agent, reacts with indole and indoleacetic acid to give purple derivatives; with skatole, indolepropionic acid and tryptophane to give brown coloured substances. In the former cases, therefore, the secondary action with trioxymethylene will not interfere with the purple colour formation, but in the latter cases the brown colour due to the formation of trioxymethylene derivatives will preponderate. The correctness of this assumption is demonstrated by the results described in Tables V *a* and V *b*. It will be noticed that a trace of trioxymethylene interferes with the appearance of the purple colour which would normally be produced under the conditions of the experiment (V *a* iii and V *b* iii). The masking effect due to the formation of trioxymethylene by the action of concentrated sulphuric acid on the formaldehyde may be obviated:

1. By using the formaldehyde solution excessively dilute, whereby the formation of trioxymethylene is delayed, and the brown colouration due to the condensation of certain indole derivatives with trioxymethylene does not interfere with the Adamkiewicz test.

2. By the addition of a trace of an oxidising agent, whereby the formation of an indole derivative, which will give the colour test, is induced.

3. In the case of tryptophane, by ensuring the previous formation of one of the three condensation products described above (p. 121).

4. By the use of glyoxylic acid as the source of formaldehyde instead

of formaldehyde itself (Hopkins and Cole's modification of the Adamkiewicz reaction).

The author suggests that at the line of junction of the concentrated sulphuric acid and the aqueous layer containing a trace of glyoxylic acid and the indole derivative, decomposition of the glyoxylic acid takes place with the liberation of formaldehyde. The vivid colour reaction may be assigned to one of the following causes:

1. The amount of formaldehyde produced under the conditions of the experiment is never more than a trace, and the corresponding trioxymethylene formation does not interfere with the colour reaction (Table III).

2. The formaldehyde is liberated in the nascent state, and is therefore intensely reactive.

Either of these explanations can be offered to account for the vivid colour reaction when the formaldehyde condensation product of tryptophane is acted upon by concentrated sulphuric acid without the previous addition of formaldehyde, glyoxylic acid or an oxidising agent. But that the nascent condition of the formaldehyde is not essential to the colour reaction is deduced from the fact that the addition of a 1/10 solution of formalin in water to the ether oxidation substance or the glyoxylic condensation product gives rise in the presence of concentrated sulphuric acid, to the formation of a purple colour.

3. In the glyoxylic reaction with tryptophane, the colour reaction is probably influenced by the action of the formaldehyde, liberated from the glyoxylic acid, on the glyoxylic condensation product formed with tryptophane.

Section II.

The following experiments show that the colour reaction induced by the action of concentrated sulphuric acid on solutions of indole derivatives to which formaldehyde and an oxidising agent have been added, is of a much more complex nature than when formaldehyde alone is used.

In Section I (p. 121) it was demonstrated that the so-called "glyoxylic" colour reactions could be obtained by the action of concentrated sulphuric acid on solutions of tryptophane and skatole to which had been added traces of indolealdehyde and formaldehyde. Therefore in the Adamkiewicz test the part played by the oxidising agent is to convert the tryptophane and skatole into some indole derivative which will react with formaldehyde to form compounds capable of giving purple colour reactions with sulphuric acid and not to oxidise the formaldehyde as Dakin suggests.

A study of the colour reactions of indolealdehyde was made:

Colour reactions induced by the action of concentrated sulphuric acid on solutions containing:

Colour produced.

(1) Indolealdehyde.

Pink. Colour becomes more intense on warming [Ellinger and Flamand, 1909].

(2) Indolealdehyde + one drop of a 1/100 solution of formalin.

Reddish purple.

(3) Indolealdehyde + trace of trioxymethylene.

„   „

The addition of an oxidising agent causes the colour to become more blue.

(4) Indolealdehyde + a trace of an oxidising agent.

Bluish magenta.

(5) Indolealdehyde + tryptophane.

Orange red colour.

(6) Indolealdehyde + tryptophane + a trace of formaldehyde (1/100).

"Glyoxylic" colour at the line of junction of the acid and aqueous solution. Above this is seen an orange red colour.

(7) Indolealdehyde + tryptophane + an oxidising agent.

Magenta.

(8) Indolealdehyde + tryptophane + a trace of trioxymethylene.

"Glyoxylic" colour as in (6) but it is rapidly masked by the brown colour characteristic of the action of trioxymethylene on tryptophane.

(9) Indolealdehyde + skatole.

Reddish pink colour.

(10) Indolealdehyde + skatole + a trace of formaldehyde (1/100).

At the junction of the acid and solution a reddish purple colour is obtained (as in (11)). Above this layer the colour is reddish pink.

(11) Skatole + a trace of an oxidising agent + a trace of formaldehyde (1/100).

Reddish purple.

If the indolealdehyde in (10) be present in excess then the colour produced is practically that produced in (9), the purple being masked.

It is obvious from these experiments that where colour reactions are produced from tryptophane in the presence of an oxidising agent the secondary reactions with indolealdehyde have to be considered, and in these cases the actual shade of colour will depend upon the conditions of the experiment.

From (6) it will be seen that the "glyoxylic" colour is produced by the action of formaldehyde (*without the addition of an oxidising agent*) on the mixture of tryptophane and indolealdehyde. It may be that the indolealdehyde reacts with the amino-group of tryptophane to form a substance which then combines with formaldehyde as did the three condensation products (Section I, Table I).

An inspection of reactions (4) and (7) shows that in the presence of an oxidising agent concentrated sulphuric acid causes indolealdehyde to condense with more indolealdehyde (4) or with tryptophane (7) to form substances giving characteristic colour reactions which are not those of the "glyoxylic" reaction.

Although the chemical reactions involved in the actual process of the purple colour formation are at present unsolved yet it is clear that the condensation of indole nuclei is an essential feature of the reaction. In the case of those compounds which give the purple colour directly with formaldehyde the condensation takes place through the agency of the formaldehyde alone. But for those substances for which, on account of interference with the colour reaction due to the formation of trioxymethylene, an oxidising agent is used, the reaction also involves the condensation of indolealdehyde with the original indole derivative taken.

Summary.

The formation of coloured condensation products from indole derivatives and certain aldehydes necessitates the use of condensing agents. The reaction takes place between the $-NH$ group of the indole nucleus and the $-CHO$ group of the aldehyde.

The evidence adduced in this paper is favourable to the Adamkiewicz reaction being primarily a formaldehyde reaction.

1. The formaldehyde condensation product of tryptophane gives the colour test directly with concentrated sulphuric acid, without the addition of an oxidising agent or of glyoxylic acid or of formaldehyde.

2. A trace of formaldehyde, without the addition of an oxidising agent, gives the colour reaction with indole derivatives.

3. When formaldehyde is used in testing for indole derivatives, the purple colour is often masked on account of further reactions taking place between the indole substance and the trioxymethylene formed from formaldehyde by the condensing power of the concentrated acid.

4. In Hopkins and Cole's modification of the test, glyoxylic acid reacts by virtue of its decomposition into formaldehyde, and for one of the two following reasons is the best reagent to use for the reaction:

(a) the formaldehyde is liberated in such small quantity that the formation of trioxymethylene does not take place to any appreciable extent.

(b) the formaldehyde so liberated is in the nascent state, and therefore more reactive.

5. In the case of tryptophane the colour test is often performed with formaldehyde in the presence of an oxidising agent. The part played by the latter is primarily to produce some oxidation product of tryptophane, e.g. indolealdehyde.

The reaction under these conditions is of a complex nature as, besides the

effects described above, (2) and (3), it involves the formation of coloured substances by the action of the concentrated acid on:

(*a*)   indolealdehyde and tryptophane.

(*b*)   indolealdehyde in the presence of an oxidising agent.

(*c*)   indolealdehyde and tryptophane in the presence of an oxidising agent.

(*d*)   indolealdehyde and tryptophane in the presence of formaldehyde.

REFERENCES.

Adamkiewicz (1874), *Arch. ges. Physiol.* **9**, 156.
Dakin (1909), *J. Biol. Chem.* **11**, 4, 289.
Ellinger and Flamand (1909), *Zeitsch. physiol. Chem.* **62**, 276.
Homer (1913), *Biochem. J.* **7**, 101.
Hopkins and Cole (1900), *J. Physiol.* **27**, 418.
————— (1902), *J. Physiol.* **29**, 451.
Rosenheim (1906), *Biochem. J.* **1**. 233.
Voisenet (1905), *Bull. Soc. Chim.* (3), **33**, 1198.

XII. THE RÔLE OF GLYCOGEN, LECITHIDES, AND FATS IN THE REPRODUCTIVE ORGANS OF ECHINODERMS.

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In the course of a research upon the protamines and histones of the spermatozoa of *Echinus esculentus* in which the reproductive organs (gonads) were extracted with hot alcohol, we were struck by the enormous amount of fatty matter removed by the hot alcohol. This separated out in drops and formed a thick layer at the bottom as the alcohol cooled. This appeared to us an unusual result to obtain from a male gland possessing only a reproductive function. It was still more surprising to find the aqueous extract made later on, in the process of extraction of the protamines, heavily laden with glycogen as shown by a marked pearly translucence, and confirmed by a marked iodine reaction even after manifold dilution. In both of these respects, the extracts of the gonads might well have been those from a liver or hepato-pancreas in a vertebrate or higher invertebrate, so rich were they in the usual products of metabolism. In the echinodermata, the gonads form by far the preponderating part of the soft tissues of the animal, and contain at least three-fourths of the organic matter of the organism. Also there exists in these animals no structure resembling a liver, or other metabolic gland.

It was accordingly determined to make analyses of the fatty constituents and glycogen of the gonads to elucidate their functions in this respect. For this purpose experiments were instituted to observe the effects of seasonal variation in April and in August—the period of sexual activity being in April; to keep the animals in batches for some weeks in the fed and unfed conditions respectively; and to test whether the gonads contained any diastatic enzymes.

The exceedingly slow metabolism of these organisms during even a lengthy period in which they were unfed, precluded us however from observing whether the reserve stores of fat and glycogen of the gonads could

be utilized for the purposes of the general metabolism of the animal as a whole. As is shown in another research [Moore, Edie, Whitley and Dakin, 1912, p. 287] upon the metabolism of marine organisms, the amount of organic matter required in twenty-four hours by an average-sized echinus is only 4·2 milligrams, and as the gonads contain about five grams of dry organic matter it is impossible to say whether these are acting as reservoirs for the minimal demands of the organism during deprivation of food even for a month or more. For example, 4·2 milligrams of organic matter oxidized daily, is equivalent to 30 milligrams of moist weight, and for one month, or 30 days, this would amount to a loss in weight of only 0·9 g. This much, however, is certain, that the gonads are very retentive of their stored-up food supplies, such as fats and glycogen, for after even three weeks' complete deprivation of food, the gonads are still very rich in both oils and glycogen, and even quantitative determinations show no constant difference between the fed and the unfed organisms. The unfed specimens are alive and well at the end of the period and commence at once to feed greedily when offered suitable food such as ordinary laminaria.

It is not to be supposed that the metabolism of the echinus is so excessively slow under normal conditions; this is only a dormant condition which can be assumed when there is necessity for it. When the organisms are first placed in captivity without food, there is an enormous output of faeces for the first two days, afterwards the water in the tank is practically free for the rest of the period. The animals also develop an appetite, which was shown by an attack upon the wooden bottoms of the tanks in which the unfed echini were stored. The unfed animals supported themselves in the stream of running water by the tube-feet just like the fed ones and appeared quite active and well in every respect.

The echini fed in the laboratory upon laminaria and other sea-weeds, ate quite vigorously and produced a large output of faeces daily, but at the end of the period, the extracts from the two sets of gonads, of fed and unfed, were very much alike in storage content. There may have been, however, an increase in bulk of the gonads, of those which were fed, for the size of the gonads varies considerably and they cannot be weighed at the beginning and end of the experiment.

Another important point to remember is that there is no trace whatever to be detected of a diastatic enzyme.

A gonad rich in glycogen may be left for some hours either at air temperature, or heated in a bath to 35°, for the same time, and at the end not a trace of sugar is obtainable.

It may even be left for a period of two days with the same result. But, extraction at the end shows the glycogen to be present in large amount, and on boiling with dilute acid a copious reduction with Fehling's solution is at once obtained.

The diminished metabolism during fasting, compared with the rapid uptake of food while fed, the persistence of fat and glycogen after long starvation, and the absence of a diastatic enzyme, when all considered together, and in conjunction with the relatively huge size of the reproductive organs, indicate that the stored fat and glycogen are intended for the internal metabolism of the gonads themselves and to subserve the enormously developed reproductive functions.

The main bulk of the metabolized food would also appear to be devoted to the nourishment and upbuilding of the reproductive organs. These organs appear during the resting season to build up rich nutritive material and then discharge this during the active season, when a diastatic ferment may perhaps be temporarily developed.

It is remarkable that a few months after the active season, as in August (the period of activity having been the end of March and earlier part of April), the gonads are as large and full as before activity, and richly charged with oils and glycogen. While being fed the uptake of material must be many times the amount required for locomotion and other physiological purposes in the unfed condition, as shown by the amount of oxygen used daily by the unfed animal. If it may be assumed that the unfed animal requires about as much energy for these purposes as the fed animal, as would seem to be shown by the activity of the two being similar, then it may be concluded that at least nine-tenths of the energy of the food taken up by the echinus is conserved for reproductive purposes.

In regard to the carbohydrate and fatty metabolism, there is no noticeable difference between the male and female gonads, both are rich in oils and in glycogen.

A considerable amount of lecithides was in all cases found admixed with the fats. The iodine value for both lecithides and oils was high, showing a marked degree of lack of saturation. In this respect the fats of the echinus resemble the fish-oils, or fish liver-oils.

Thus, for the oils, iodine values varying from 130 to 190 were obtained, and for the lecithides, values of 70 to 120. These high iodine values suggest that these fatty constituents may possess therapeutic values similar to those of cod-liver oil in wasting diseases.

Methods of Extraction and Analysis.

In the earlier experiments carried out at Easter 1911, only the fats, oils and lecithides as extracted by thorough treatment (*a*) with cold and (*b*) with hot alcohol afterwards, were obtained and analysed.

The work was resumed in August and September 1911, and Easter 1912, and then two methods chiefly were employed. In the first method the glycogen only was estimated, by boiling out the gonads twice with water acidulated with acetic acid, after which a third decoction showed no opalescence and gave very little reaction with iodine. The two extracts were added together, precipitated by the double volume of alcohol, washed with 70 per cent. alcohol by decantation, and preserved in this for some months, after which the precipitated impure glycogen was digested with strong caustic potash, as in Pflüger's method described in the concluding part of the second method below, followed by precipitation with alcohol, washing with alcohol, drying and weighing. In several cases, the amount of glycogen present was finally determined by hydrolysis to glucose, reduction by excess of Fehling's solution, and gravimetric determination of the CuO. When this was done it was found that the product of the Pflüger's method was never pure glycogen, but varied from 63 to 93 per cent. pure.

Both results are given alongside each other in the tables below for comparison. The nature of the impurity was not determined, but it indicates that this method of determining glycogen should be used with caution, and always followed by a hydrolysis and sugar determination.

Second Method. In this, the final method used, which was found by far the most convenient, oils, lecithides, and glycogen were all determined in the same specimen. At the end of the preliminary treatment of the organisms at the Biological Station, the shells were broken open by chipping round in a sectional plane midway between mouth and anus, and the sex determined by examining a smear from a gonad with a low power of the microscope.

The echini were thus sorted into two heaps, the five gonads were removed from each echinus, and two heaps made of male and female gonads respectively. The weight of each quantity in the fresh condition was taken, and they were then broken up and placed in about three times their volume of absolute alcohol. In this condition they were transported to Liverpool and after some months were then extracted for oils and lecithides, and for glycogen respectively by the following method.

The alcohol was decanted and filtered off, without previous heating. A

fresh quantity of absolute alcohol was then added to the tissue which was previously broken up as finely as possible, and mechanical agitation in a bottle on a shaker was used for some hours. This second quantity of alcohol was filtered off as before, and when mixed with the first, the mixture formed the " cold alcoholic extract."

The " hot alcoholic extract" was obtained similarly by two extractions with boiling alcohol, decantation and filtration.

Each alcoholic extract was subsequently treated in precisely the same fashion for determination (a) of salts and extractives, (b) lecithides, and (c) fats and oils, as follows:

The alcoholic extract was evaporated till all alcohol had been removed, and the total weight was taken. The residue was exhausted by repeated portions of methylated ether, which portions were all united. The residue from the ether consisted of salts, creatine, etc., and this was weighed separately. The ethereal extract was evaporated down cautiously to a thin syrupy consistency, acetone in excess was then added to precipitate the " lecithide " fraction, this was dried and weighed and constants determined. The portion soluble in acetone gave the product described under the heading oils and fats. It is, of course, only claimed that this is a rough separation. The labour of a complete separation in so many experiments would have been inordinately great, and the figures for the constants in the two respective columns (1) lecithides, and (2) oils and fats, show that these two fractions represent a fairly complete separation of the lecithides from the oils. The weight, saponification value, and iodine value, were determined in each case.

The dried tissue after removal of fats, etc., as above described, was weighed and then digested for three hours on a steam bath with 40 per cent. solution of caustic potash, which, as discovered by Pflüger, leaves the glycogen intact while it decomposes proteins. After cooling, twice the volume of absolute alcohol was added, so precipitating the glycogen. The glycogen was filtered off through a Gooch crucible provided with a filter of finest woven silk bolting such as is used for flour sifting. This was found to be much preferable to glass-wool, or asbestos, and may be recommended as a filtering medium for alkaline fluids. The precipitate is then washed with a 15 per cent. solution of caustic potash in a 66 per cent. solution of alcohol, as recommended by Pflüger. Next it is washed through into solution in a fresh receiver with hot water. The aqueous solution is again filtered, reduced in volume by evaporation if necessary, and finally precipitated by twice its volume of absolute alcohol, filtered, washed with two-thirds alcohol, dried and weighed. Finally, the content of glycogen in the product is determined

TABLE I.

Experiments of Easter 1911. Determinations of Lecithids and of Fats and Oils in Ripe Ova and Spermatozoa of Echinus esculentus.

Material and extractive	Total weight of extract	Salts, creatine, etc. extractives	Lecithide values			Fat and Oil values			Dry weight of residue after complete alcoholic extraction
			Total weight	Saponification value	Iodine value	Total weight	Saponification value	Iodine value	grams
OVA.									
Exp. I. Ripe Ova, 580 g. moist weight:									
Cold alcohol ...	8·56	2·90	2·10	259	73·4	2·85	205	166	27·95
Percentages on moist weight ...	1·48	0·50	0·36	—	—	0·49	—	—	
Hot alcohol ..	11·44	0·35	2·34	207	76·0	8·13	192	136	
Percentage ...	1·97	0·06	0·40	—	—	1·40	—	—	
Exp. II. Ripe Ova, 352 g. moist weight:									
Cold alcohol ...	7·10	3·50	1·10	207	84·0	2·15	188	133	12·05
Percentage ...	2·14	1·06	0·33	—	—	0·65	—	—	
Hot alcohol ...	8·10	1·20	1·70	229	82·0	4·23	195	145	
Percentage ...	2·44	0·36	0·51	—	—	1·30	—	—	
SPERMATOZOA.									
Exp. III. Ripe Spermatozoa, 700 g. moist:									
Cold alcohol ...	9·04	1·04	2·60	176	86·1	4·61	201	140	21·7
Percentage ...	1·29	0·15	0·37	—	—	0·66	—	—	
Hot alcohol ...	10·62	0·72	0·50	180	77·0	9·08	172	131	
Percentage ...	1·52	0·10	0·07	—	—	1·30	—	—	
Exp. IV. Ripe Spermatozoa, 235 g. moist:									
Cold alcohol ...	3·10	0·48	0·65	186	90·7	1·40	200	166	(Not taken)
Percentage ...	1·31	0·20	0·28	—	—	0·60	—	—	
Hot alcohol ...	7·67	0·75	1·80	190	81·0	3·33	197	153	
Percentage ...	1·28	0·32	0·77	—	—	1·43	—	—	

by hydrolysis, and gravimetric determination by precipitation in excess of Fehling's solution.

Experiments of Summer 1911 (*August and September*). The first four experiments to be described in this series were in regard to content of glycogen only, and the effects of lack of food upon the glycogen content of the gonads. The gonads in all these experiments are taken at an inactive sexual period. The ova in the females at this time of year are unripe and small, and formed eggs are small in number relatively to other tissue; the spermatozoa in the males are alive and moving, but small.

Thirty echini of medium and large size only were taken from Port Erin Breakwater at low tide on August 22, all were kept in a large tank filled with running sea-water until August 24 (about 48 hours), not being fed in the interval.

Twenty of the echini were then opened and the gonads examined for sex, twelve were found to be females and eight were males. The female and male gonads separately were at once treated for glycogen separation as will presently be described in Expts. V and VI. The remaining ten echini were kept in the tank in fresh running sea-water without any food until Sept. 11, that is for a period of twenty days in all; they were alive and well at the end of that period, and were then opened, when gonads, and all the other organs, presented quite a healthy and well-fed appearance: the intestines were of course empty. On examination of these for sex, four were found to be females and six were males. The two masses of female and male gonads were then at once treated for extraction of glycogen in the fresh condition with the results shown in Expts. VII and VIII.

Experiment V. The gonads from the twelve females described above weighed 440 g. in the moist condition or an average of 36·6 g. per animal. As subsequent determination of dry weight of a given weight of moist gonad gave a solid content of 17·2 per cent., this weight represents 75·7 g. of dry solid. This dry solid has salts present in the proportion of sea-water in invertebrates so that the amount of dry organic matter is about 62·5 g.

The material was mashed up into a soft pulp, and the proteins coagulated by dropping the pulped mass into 400 c.c. of boiling one per cent. acetic acid in distilled water, and boiling for five minutes. The fluid was then filtered off through fine silk gauze and pressed out. The residue of tissue was pounded ·up and extracted with 500 c.c. of distilled water in two portions. The last filtrate was almost clear of glycogen, and gave only a trace of reaction with iodine. The first filtrate in dilute acetic acid was a strongly opalescent solution but without ·obvious suspension. This was again filtered through

two folds of fine silk, and a small portion of the filtrate which was still strongly opalescent gave a strong port-wine colour with iodine, and on boiling for five minutes with 3 per cent. hydrochloric acid gave a copious reduction with Fehling's solution.

The main volume of the acetic acid solution and the washings were united and precipitated with twice the volume of absolute alcohol giving an abundant white precipitate settling easily. This was washed, after decantation, with more two-thirds alcohol, and carried under alcohol to Liverpool, where it was later passed through the Pflüger process for purification of the glycogen and analysed. The weight of glycogen obtained was 7·44 g., but hydrolysis and a gravimetric Fehling determination showed this to be only 79 per cent. pure glycogen giving 1·33 per cent. glycogen on the moist weight, or the glycogen in the gonad is 9·36 per cent. of the total organic matter.

Experiment VI. The eight sets of male gonads weighed 258 g., thus averaging 32·25 g. per animal. Of this quantity, 200 g. were taken for glycogen extraction, corresponding to 28·2 g. of dry organic matter. The tissue was put through exactly the same process as used for the female glands, and gave the same appearances and the same qualitative tests. The filtration of the dilute acetic acid solution was more difficult, probably on account of the presence of the minute spermatozoa, but obviously there was much glycogen present, as shown by fine opalescence and iodine test. After filtration through fine silk it was precipitated by two volumes of alcohol as before, and carried off for analysis. Any spermatozoa present in the original acetic acid precipitate would be destroyed by the subsequent boiling with strong alkali in the Pflüger process. The amount of glycogen obtained was 3·02 g., and this was found on hydrolysis to be 81·4 per cent. pure, corresponding to 1·51 per cent. of crude and 1·23 per cent. of pure glycogen in the moist gonad, or to 8·66 per cent. of the total organic matter.

It is thus seen that the percentage of glycogen is much the same in the male and female gonads, corresponding to 9·36 per cent. in the female and 8·66 per cent. in the male of the dry organic matter.

Experiment VII. The four sets of gonads from the females which had been kept unfed in the tank for 20 days weighed 122 g., or an average of 30·5 g. per echinus, while Expt. V shows that the twelve sets of gonads of females killed without previous abstention from food weighed 440 g., an average of 36·6 g. The difference is not great and is probably quite fortuitous, as the content of glycogen indicates no effects of the twenty-days' fast.

The process of extraction was exactly the same as in Expts. V and VI,

and yielded similar qualitative results. The yield of crude glycogen was 2·09 g. but this, for some unknown reason, showed only 62 per cent. of glycogen on hydrolysis, corresponding to 1·06 per cent. of the moist gonad, or 7·47 per cent. of the total organic matter. This is a somewhat smaller figure but the decrease is too small to mean any real demand on the gonads for reserve food by the rest of the organism during the period of inanition, also they are still very rich in glycogen. Moreover, the result is the reverse in the experiment with the males now to be described.

Experiment VIII. The six sets of gonads from males kept without food in the tank of running sea-water for 20 days weighed 237 g., thus averaging 39·5 g. per animal. These were extracted with dilute acetic acid and precipitated with alcohol at Port Erin on similar lines to the previous experiments, and the precipitated crude glycogen was afterwards worked up by Pflüger's method at Liverpool, and controlled by hydrolysis and estimation of glucose. There were obtained 4·45 g. of glycogen, shown by hydrolysis to be 82·8 per cent. pure ; this works out at 1·54 per cent. of pure glycogen in the moist gonad, or 10·84 per cent. of the organic matter. It is here seen that the glycogen in the unfed males is somewhat higher than in the fed males of Expt. VI ; but as the females are in the reverse direction by about a similar amount, the proper conclusion is that the four experiments demonstrate no appreciable disappearance of glycogen from the resting gonads of male or female as a result of a fast of twenty days. All four experiments concordantly show that the reproductive organs of *Echinus esculentus* are very rich in glycogen.

A second catch of 60 well-grown individuals was made from Port Erin Breakwater on August 25 at low water with which the remaining experiments of this series were carried out. All these were left over-night in a large tank through which there ran an abundant supply of fresh sea-water. Next morning a large amount of faecal matter obviously chiefly of vegetable origin was found on the bottom of the tank. On the morning of the day after capture (Aug. 26), 15 individuals taken indiscriminately were opened, examined for sex, and the gonads assembled in two lots, one male, the other female. Out of these 15 specimens, six were females, eight males, and one was rejected as being in an abnormal condition. The six female sets of gonads weighed 154 g., or 26·6 g. per individual ; the eight male sets weighed 235 g., or 29·4 g. per individual. The mass of gonads in each case was sampled out into three portions which were used as follows : (1) was at once placed directly in alcohol, for determination later of oils and fats lecithides, and glycogen, (2) was at once extracted with dilute acetic acid and

precipitated with alcohol, etc. for glycogen determination, as in the preceding four experiments, and (3) a smaller quantity was dried on a bath for determination of total solid matter in the fresh tissue of the gonad.

Out of the 154 g. of female gonad, 74·5 g. were placed in alcohol (see Expt. IX b), 61·2 g. were extracted fresh with dilute acetic acid (see Expt. IX a), and 14·26 g. were taken for determination of total solids, which were found to amount to 17·2 per cent., as the glands contain approximately 3 per cent. of inorganic salts, on the basis that their fluids are isotonic with sea-water this leaves 14·2 per cent. of dry organic matter in the female gonad.

Similarly the eight male gonads weighing 235 g. were divided into 75·1 g. placed directly in absolute alcohol (see Expt. X b), 134·4 g. extracted fresh with dilute acetic acid (see Expt. X a), and 12·75 g. taken for determination of total solids. The percentage of total solids found was 16·8, and after deduction for inorganic constituents this leaves 13·8 per cent. of dry organic matter in the tissue of the male gonad. This lies very close to the figure for the female organ given above, and the values of 17 per cent. for total solids, and 14 per cent. for organic solids, may be taken as an average.

The remaining 45 individuals of the second catch (Aug. 25) were used, as follows, for experimentation in the laboratory. They were divided into three lots of 15 individuals each, the sizes being arranged as far as possible so as to give no advantage to any one batch[1]. The first batch were left unfed in the large tank (see Expts. XI and XII). The second and third batches were removed in order to have plenty of room and fresh running sea-water, to a series of smaller tanks forming part of a fish-hatching apparatus not at the time in use. The thirty echini were distributed over ten of these tanks, three in each tank. Fifteen of these echini were fed on fresh laminaria gathered on the shore (see Expts. XII and XIII) and they ate a surprisingly large amount of it daily. The other fifteen, forming the third batch, received no food of any kind during the period of the experiment (see Expts. XIV and XV). As some index of the rate of feeding of the echini supplied with laminaria there may be mentioned the amount of faeces found in the tanks.

The faecal masses were washed into a graduated 100 c.c. measure, allowed to sediment, and the volume of the sediment measured. The volumes found were as below. At this time in the experiment a few of the echini had perished, as noted below :

Tank No. I. Two echini, 45 c.c. of faecal sediment in three days.

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[1] As a matter of fact it frequently happens that heavier gonads are obtained from a medium-sized than from a large echinus, so that this precaution is scarcely required so long as no individuals below a certain size are taken in the first instance.

Tank No. II. Two echini, 35 c.c. of faecal sediment in three days.

Tank No. IV. Three echini, 100 c.c. of faecal sediment in three days.

The amount of laminaria eaten was not actually weighed, but it was quite a surprising amount for organisms of so slow a metabolism, and was evidently chiefly being used for storage in the gonads. The unfed batches of these echini produced no faeces, after the first two days or thereabouts, and at the end of the experiment it was noticed that in the tanks of the fish-hatching apparatus, which were made of planed wood, in each case four or five bitten out spots showed where the animals had attacked the wood of the tank floor and scooped it out quite distinctly; no similar attack had been made on the tanks containing those fed on laminaria. The experiment lasted from August 25 till Sept. 13, a period of nineteen days. At the end the gonads were removed, examined for sex, and placed at once in absolute alcohol, from which they were taken and analysed some months later in Liverpool.

Early in this experiment a certain number of the echini in each of these three batches succumbed. This result was in no wise due to lack of food, for as great a percentage died in the fed batch as in the corresponding unfed batch. Such mishaps often occur soon after the echini have been taken, for some unknown reason, and then the survivors keep in quite good condition. All the animals alive at the end were active and appeared quite normal and healthy. Out of the fifteen kept in the large tank, nine survived, of which six were males and three were females; of the fifteen fed daily in the fish-hatching tanks, eleven survived, seven females and four males; of the fifteen kept unfed in fish-hatching tanks, eleven also survived, six males and five females.

Experiment IX a. Amount of glycogen in freshly extracted female gonads. This experiment was carried out with a portion, 61·2 g., of the gonads of females of Catch II, freshly extracted with dilute acetic acid. Details as before, amount of glycogen 1·14 g., found on hydrolysis 87·7 per cent. pure = 1·63 per cent. of pure glycogen in fresh gland, or 11·64 per cent. of the dry organic matter.

Experiment IX b. Determinations of (*a*) lecithides, (*b*) fats and oils, and (*c*) glycogen in a portion, 74·55 g., of female gonads of Catch II, placed at once in alcohol. Hot and cold alcoholic extracts were taken and analysed as described, and the glycogen determined in the residue. In percentages of the moist tissue, the following were the results obtained: glycogen, 1·53 per cent.; lecithides, cold alcohol 0·41, hot alcohol 0·25, total = 0·66 per cent.; constants (cold alcohol), sapon. value 210, iodine value 78·0; (hot alcohol), iodine value 91·1. Fats and oils, cold alcohol 0·4, hot alcohol 1·46, total =

1·86; constants (cold alcohol), sapon. value 208, iodine value 180; (hot alcohol), sapon. value 197, iodine value 174.

Percentages expressed on dry organic matter: glycogen, 10·9 ; lecithides, 4·6 ; fats and oils, 13·1.

Experiment X a. This was carried out upon a portion, 134·4 g., of the male gonads of Catch II, freshly extracted with dilute acetic acid, etc. Amount of glycogen obtained, 2·66 g. of 81·15 per cent. purity = 1·60 per cent. of glycogen in fresh gland, or 11·3 per cent. of the dry organic matter.

Experiment X b. Determinations of (*a*) lecithides, (*b*) fats and oils, and (*c*) glycogen in a portion, 75·1 g., of male gonads of Catch II, placed at once in alcohol. Details as in Expt. IX b. Results obtained in percentages of moist tissue: glycogen, 1·70 ; lecithides, cold alcohol, 1·01, hot alcohol 0·26, total = 1·27 ; constants of lecithides, (cold alcohol), sapon. value 240, iodine value 89, (hot alcohol), sapon. value 197, iodine value 98·2 ; fats and oils, cold alcohol 0·54, hot alcohol 1·80, total = 2·34 ; constants of fats and oils, (cold alcohol), sapon. value 206, iodine value 160, (hot alcohol), sapon. value 202, iodine value 181.

Percentages expressed on dry organic matter: glycogen, 11·90; lecithides, 8·89 ; fats and oils, 16·38.

Attention may again be drawn to the highly unsaturated nature of the fats and oils as shown by the high iodine values, in both male and female glands, and to the high percentages of reserve storage materials throughout. There was also a large amount of organic matter soluble in 66 per cent. alcohol, and a relatively small amount of insoluble protein and tissue debris, but figures for these are not given, as analyses of the portions were not made. The results were similar in this respect to Table II in which the total amount of salts and alcohol soluble extractives are shown in a similar set of experiments.

Experiments XI to XVI. The whole of the glands in each of these experiments were placed at once, after conversion into a soft pulp, in twice their volume of alcohol. Through a misunderstanding they were used for the glycogen determinations after having been extracted with cold alcohol only, so that the figures given below do not represent total amounts of lecithides, or fats and oils. They are comparable amongst each other, however, and show that there is no appreciable diminution of fatty substances as a result of the nineteen-days fast.

Experiments XI and XII refer to the unfed echini in the large tanks; there were three females, weight of gonads 73 g., average 24 g., and six males, weight of gonads 152 g., average, 25 g. Experiments XIII and XIV refer

TABLE II.

Portion of Experiments of Summer 1911. Determinations of Lecithides, Fats and Oils, and Glycogen in Unripe Ova and Spermatozoa of Echinus esculentus. The experiments show results, in animals kept, fed and unfed, during a period of 19 days in laboratory (Port Erin I.O.M.).

Nature of material extracted with cold alcohol	Total weight of extract	Salts, creatine, nitrogenous bases, etc.	Lecithide values			Fat and Oil values			
			Total weight	Saponification value	Iodine value	Total weight	Saponification value	Iodine value	Glycogen
Exps. XI and XII. **Unfed** animals together in large tank.									
Exp. XI. Ova unfed:									
Weights in 73 g.	11·1	8·90	1·16	181	100	0·74	203	186	0·56
Percentages of moist tissue ...	15·2	12·19	1·59	—	—	1·01			0·77
Percentages of dry organic matter	—	—	11·13	—	—	7·07			5·39
Exp. XII. Spermatozoa unfed:									
Weights in 152 g.	13·55	10·25	1·66	225	125	1·5	230	170	2·41
Percentages of moist tissue ...	8·91	6·74	1·09	—	—	1·0			1·59
Percentages of dry organic matter	—	—	7·63	—	—	7·0			11·13
Exps. XIII and XIV. **Fed** animals in fish-hatching tanks.									
Exp. XIII. Ova fed:									
Weights in 204 g.	14·1	11·20	1·88	187	112	0·85	220	180	2·44
Percentages of moist tissue ...	6·9	5·49	0·92	—	—	0·41			1·20
Percentages of dry organic matter	—	—	6·44	—	—	2·87			8·40
Exp. XIV. Spermatozoa fed:									
Weights in 118 g.	9·30	6·90	1·37	212	118	0·95	209	191	1·20
Percentages of moist tissue ...	7·90	5·85	1·16	—	—	0·80			1·02
Percentages of dry organic matter	—	—	8·12	—	—	5·60			7·11
Exps. XV and XVI. **Unfed** animals in fish-hatching tanks.									
Exp. XV. Ova unfed:									
Weights in 173 g.	13·5	9·85	2·11	189	108	1·20	227	166	0·51
Percentages of moist tissue ...	7·8	5·69	1·22	—	—	0·69			0·30
Percentages of dry organic matter	—	—	8·54	—	—	1·83			2·10
Exp. XVI. Spermatozoa unfed:									
Weights in 178 g.	14·01	10·5	1·20	181	101	1·95	189	152	2·00
Percentages of moist tissue ...	7·88	5·9	0·67	—	—	1·10			1·13
Percentages of dry organic matter	—	—	1·69	—	—	7·70			7·91

to echini fed during these 19 days with laminaria, but kept otherwise under strictly similar conditions to those in the two subsequent experiments (Expts. XV and XVI); there were seven females, weight of gonads 204 g., average 29 g., and four males, weight of gonads 118 g., average 29 g. Experiments XV and XVI refer to echini unfed, but otherwise similar to those in Expts. XIII and XIV; there were five females, weight of gonads 173 g., average 34 g., and six males, weight of gonads 178 g., average 29 g. The average total weight of gonads is less in the case of the unfed echini in the large tank, but in those unfed, alongside the fed, in the fish-hatching apparatus no such effect is discernible. In fact the gonads of the unfed females are appreciably heavier than those of the fed females, while the males are practically of equal weight in the two series. The only conclusion valid is that even a nineteen-days period of removal of supplies is ineffectual to cause any real starvation, or appreciably use up reserves.

It would certainly have been expected from the amount of laminaria used by the echini that some increase of weight might have taken place in the fed animals, but it is possible that only a small amount of the nutritive organic matter of the laminaria was actually utilised by the echini. Certainly the faeces still contained a large amount of nutrition for they were swarming with copepoda when examined microscopically. These no doubt came in with the water supply and remained and accumulated upon the faeces of the echini, which formed their food supply.

The analytical results of these six experiments are shown in Table II. The figures obtained in these experiments show no clear evidence of diminution of reserves in the glands as a result of stopping the feeding for nineteen days, for there is still abundance of glycogen and fatty materials.

Experiments of Easter 1912. The echini used in these experiments were taken a week or two after the active sexual period which appears to have been somewhat early in this particular Spring. At least it was difficult to obtain any ripe eggs for fertilisation about the period of this experiment. The catch was made on April 10 and was divided into two lots, the animals of one of which were kept unfed from April 12 till April 21, while those of the other lot were fed on laminaria. The animals were killed at the end by opening the shells, examined for sex, and separated into four heaps, male and female, fed and unfed, respectively. These were immediately fixed by placing in two volumes of alcohol and taken to Liverpool for the analyses, which were conducted as described above; the results are given in Table III. It will be observed that the results do not differ materially from the others. Only percentage figures and the constants are given in the table.

TABLE III.

Organs extracted	Lecithides			Oils			Glycogen
	Percentage of dry organic matter	Saponification value	Iodine value	Percentage of dry organic matter	Saponification value	Iodine value	Percentage of dry organic matter
Exp. XV. Sperm. of fed males, wt. 205 g.							
Cold alcohol	5·1	159·6	86·0	4·1	164·7	150·1	—
Hot alcohol	7·2	176·1	90·1	5·0	122·3	133·4	—
Total	12·3	—	—	9·1	—	—	7·6
Exp. XVI. Ova of fed females, wt. 171 g.							
Cold alcohol ...	2·87	168·1	82·8	3·15	178	134·7	—
Hot alcohol ...	3·92	172·8	89·7	3·92	185·1	129·5	—
Total	6·79	—	—	7·07	—	—	6·2
Exp. XVII. Sperm. of unfed males, wt. 62 g.							
Cold alcohol ...	9·10	163·4	94·5	10·43	158	148·6	—
Hot alcohol ...	1·12	174·3	101·0	1·12	112·1	160·6	—
Total	10·22	—	—	11·55	—	—	11·1
Exp. XVIII. Ova of unfed females, wt. 160 g.							
Cold alcohol ...	4·08	164·2	88·6	6·08	168·1	142·1	—
Hot alcohol ...	3·76	171·7	96·3	11·68	174·2	118·4	—
Total	7·84	—	—	17·76	—	—	9·1

CONCLUSIONS.

1. Both male and female reproductive glands in echinoderms contain large amounts of reserve metabolic products such as glycogen, fats and lecithides.

2. These reserves are only slowly used up, if at all, when the animal is deprived of food.

3. In a reproductive gland richly stored with glycogen, no sugar formation occurs on keeping after death, even in a period of two days.

4. The amount of food consumed is much greater than that required to cover the daily metabolic wants of the animal, and is largely stored in the reproductive glands during the resting period, but it has not yet been possible to trace the conversion of this at the active reproductive season.

5. The fatty constituents of the reproductive organs of the echinoderm are highly unsaturated, and resemble in this respect liver oils.

REFERENCE.

Moore, Edie, Whitley and Dakin (1912), *Biochem. J.* 6, 255.

XIII. THE BASIC AND ACIDIC PROTEINS OF THE SPERM OF *ECHINUS ESCULENTUS*. DIRECT MEASUREMENTS OF THE OSMOTIC PRESSURE OF A PROTAMINE OR HISTONE.

By BENJAMIN MOORE, EDWARD WHITLEY AND ARTHUR WEBSTER.

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So far as we are aware no one has hitherto made a determination of the molecular weight in colloidal solution of any member of those interesting classes of proteins with marked basic properties known as the protamines and histones. These substances have been set down by workers upon them such as Miescher and Kossel as the simplest steps in natural protein synthesis which furnish a clue as to the constitution of the others, and it is rather remarkable that no attempts have been made to study their degree of complexity in solution. The present paper must only be regarded as a tentative experiment in this direction, to be made more complete when more material becomes available; for the chief difficulty lies in obtaining suitable material and working it up into a supply of pure protamine or histone. The substance which we have isolated from the ripe male gonads of *Echinus esculentus* appears to us to stand intermediately between a protamine and a histone, for it gives feebly the Millon's test as a reddish colouration on boiling, with no precipitate; and yet it possesses all the properties of a protamine. This substance dissolved as a sulphate in distilled water gave an osmotic pressure leading to a molecular weight in solution much less than that of an albumin or globulin, the figure being approximately 8780 when calculated to standard temperature and pressure.

It behaved as a true colloid in being perfectly indiffusible through parchment paper, and the osmotic pressure rose steadily to a maximum, and remained there at a constant level. Afterwards, the sulphate of the protein

was thrown out in apparently quite unaltered form by excess of alcohol, dried and weighed. The substance comes down quite sharply with excess of alcohol, as a white precipitate readily soluble again in distilled water. As this process was repeated several times before measurements were taken, it is quite certain that if there was any admixture it must have been that of very closely allied substances of this class.

The only other substance of this protein group from the echinodermata ever examined was a substance isolated from the sperm of *Arbacia* by Matthews [1897], and called by him *arbacin*. This substance is classed amongst the histones by Matthews chiefly because it gave a positive result with the Millon's test. The substance with which we were working, from a different species of echinus, in a first preparation gave a negative reply to the Millon's reagent, and in a second preparation gave a somewhat feeble red colouration but no precipitate, and in absence of more complete analysis we are consequently somewhat doubtful as to whether we ought to classify it as a protamine or histone, so for the present we prefer to leave the point open.

It may be of some interest to pause here to state that our original intention in isolating this substance at Port Erin in the Spring of 1911, just at the commencement of the active sexual period, was to observe whether protamines and histones acted as hormones to the earlier stages of cell-division in sexual reproduction.

The whole group of the protamines is intimately connected with the male reproductive organs, being found in these glands almost exclusively in fishes, and in greatest abundance just before the sperm cells are ripe for discharge. The histones are obtained from the ripe spermatozoa of fishes, but in addition extend up to the sperm cells of mammalia. Both protamines and histones are strongly basic bodies, showing a markedly alkaline reaction when in solution as free bases, and combining strongly with acids, such as sulphuric acid, in definite molecular proportions.

Now it was shown some years ago by Moore, Roaf, and Whitley [1905] that almost infinitesimal amounts of alkali added to sea-water increased the rapidity of cell-divisions in fertilized echinus eggs, and that a slightly greater amount of alkali hurried the process into marked abnormalities.

It was accordingly thought that small amounts of alkaline proteins, such as protamines or histones, present in the sperm, ought to have prominent functions in relationship to cell-division.

It was from this point of view that the protamine, or histone, from echinus sperm was prepared and its action tested (a) upon unfertilized ripe

eggs and (b) upon fertilized ripe eggs, alongside controls developed in sea-water only.

The results were uniformly negative: whether this may be due to our being unable to realize proper experimental conditions we do not know. It may well be, that protamine added as sulphate to sea-water is in some wise rendered inert and incapable of penetrating the ovum, while the same substance carried into the ovum as part of the sperm-nucleus may be most effectual. The occurrence of these peculiar proteins in the sexual organs would seem to indicate some relationship with a reproductive process, but we have quite failed to realize an experimental proof.

The only positive result obtained by treatment of the fertilized eggs was that an extract of the crude sperm powder, after treatment with alcohol as described below, added in small amount, such as 1 in 4000, to sea-water inhibited cell-division and induced irregularities. Sperm nuclein appeared to possess a like action, but the protamine sulphate was quite inert.

The substance was separated from the male gonads by the well-known process of Kossel. About sixty well-grown echini were captured upon the breakwater at low tide on April 14th, 1911. The gonads of each individual were examined microscopically for sex identification and a mass of about 700 g. of spermatic gonads was obtained. This material was broken up finely and taken out in a large volume of water acidulated with acetic acid which causes the sperm cells to agglutinate and separate off. This was collected, extracted once with cold alcohol, and then exhausted of all fats and lecithides by four extractions with hot alcohol, and one with ether. The ether took out practically nothing on account of the previous exhaustive treatment with hot alcohol. The dried residue from all this extraction, of 700 g. of moist gland, amounted to only 21·68 g. This dried material remaining to the end, contains the protamines or histones and the nucleins or nucleic acids in combination together as nucleoproteins. To obtain the basic proteins (protamines or histones) it is extracted, still following Kossel's instructions, with five times its volume of half per cent. sulphuric acid, shaking vigorously at each extraction for about 15 minutes, followed by separation through fine silk gauze and pressing out. About three extractions were found to exhaust all protamine or histone from the material. After subsequent complete filtration through filter paper, the sulphuric acid extracts were precipitated with three volumes of absolute alcohol, yielding a nearly pure white precipitate which after standing for 24 hours was decanted and filtered by a Buchner filter. The precipitate dissolved readily in distilled water, and was purified by dissolving and reprecipitating with alcohol four times. It was

then dried after washing thoroughly with ether and weighed. The weight of the precipitate from 700 g. of moist gland, or 21·68 g. of dried tissue residue after alcoholic and ethereal extraction, was 1·95 g.

The residue from the extraction of the tissue with dilute sulphuric acid is exceedingly rich in nucleins or nucleic acid. It was freed from acid and dried by extraction with alcohol and ether, and then left to extract over night in the cold with one per cent. solution of caustic soda. When treated with the dilute alkali it swells up into a jelly which is almost solid in consistency and cannot be filtered in this condition, but it aggregates somewhat on adding three times its volume of absolute alcohol. It was left over night in this condition, but next morning was still unfilterable through paper. It was cleared of grosser debris by filtration through the finest silk bolting so giving a colloidal suspension free from large particles. When this alkaline alcoholic suspension was made just acid by addition of normal hydrochloric acid, a white flocculent precipitate was obtained which settled rapidly. This filtered quite readily leaving a very fine almost white precipitate on the paper. This precipitate was washed with distilled water over night, and until the washings were practically neutral next morning. It contained no protamine or histone as was shown by extracting it with dilute sulphuric acid and none was obtained from it at any stage, showing that the 1·95 g. mentioned above represented practically the total amount in the glands. The above precipitate caused by the acid in the alkaline extract, representing nuclein or nucleic acids, was weighed and found to be 7·45 g. A second extraction of the tissue residue separated by the silk filtration, yielded by the same method of alkaline extraction, followed by alcohol and acid, about 1·12 g. of a similar nucleic product, so that the total amount of acidic material was $7·45 + 1·12 = 8·57$ g. in combination with 1·95 g. of basic protein. The final residue after extraction with both acid and alkali was a substance which swelled up into a thick jelly in dilute alkali but did not dissolve in it, and shrank up when treated with dilute acid and was obviously an insoluble fibrinous form of protein. The weight after treating with alcohol, drying and weighing was 9·10 g. This material, calculating from the above weights, and the total weight of dried tissue extracted, is seen to form roughly half of the organic residue remaining after extraction with dilute acetic acid followed by alcohol and ether.

A second extraction of a different batch of male gonads made afterwards in Liverpool gave similar results.

A small amount of the protamine (or histone) sulphate was used at Port Erin for the investigation of effects upon cell-division, the remainder was

afterwards made up into a solution in distilled water containing approximately two per cent. of protamine sulphate. This was introduced against distilled water in the type of osmometer already described in previous papers [Moore and Roaf, 1907]. The dialysing membrane was parchment paper and complete equilibrium was established in three days' time as shown by the figures given below. At the end negative results were obtained on testing for protamine on the water-side. The protamine solution on the solution side of the membrane was quite sweet at the end of the experiment, and the protamine was precipitable from solution by alcohol as at the beginning. The solution had of course become somewhat diluted by expansion, and a determination of the amount of protamine sulphate by precipitation by excess of alcohol, filtering on to a Gooch crucible, drying by washing with alcohol and ether and weighing gave 1·65 per cent. of protamine sulphate. This gave a steady osmotic pressure of 34 millimetres of mercury at 15°, which works out at 19·3 millimetres for a one per cent. solution at 0°, and leads to a molecular weight in solution of the protamine solution of 8780.

The following is the protocol of the experiment:

Date	Time	Manometric reading	Temperature
1911. Nov. 21st	12 noon	388	15°
,, 22nd	9.30 a.m.	391	13·5°
,, 23rd	9.45 a.m.	396	14·0°
,, ,,	5.30 p.m.	397	16·5°
,, 24th	10.0 a.m.	401	15·0°
,, ,,	6.0 p.m.	403	16·0°
,, 25th	9.45 a.m.	406	14·5°
,, ,,	5.45 p.m.	406	15·5°
,, 26th	10.0 a.m.	406	14·5°
,, ,,	5.30 p.m.	406	15·5°
,, 27th	9.30 a.m.	406	15·0°
Opened. Zero Pressure		389	

(Constant, spanning the readings of 406)

Osmotic Pressure = 2 (406 − 389) = 34 millimetres of mercury.   Percentage of protamine sulphate = 1·65.

As has clearly been shown by Moore and Roaf, and confirmed by Lillie and others, the molecular weight of colloids in solution varies with the solvent, amounts of neutral salts, and acidity or alkalinity. Such reagents affect the colloidal aggregation. Still it is of interest to compare the complexity of solution aggregation of this basic protein, with averages for other proteins, and colloids.

The following results have been obtained by various observers:

Colloid	Osmotic pressure for one per cent. of Colloid	Molecular weight in solution
Gelatin (Moore and Roaf)	9·2 mm.	18,500
Egg albumin (Moore and Parker) ...	13·1	13,000
Serum (,, ,,) ...	2·8	60,800
Alkalized Serum (,, ,,) ...	17·0	10,000
Sodium Oleate (,, ,,) ...	10·4	16,300
,, Palmitate (,, ,,) ...	10·0 ·	17,000
,, Stearate (,, ,,) ...	9·4	18,000
Caseinogen (Moore, Roaf and Webster) ...	129·0	1,400
Haemoglobin (Hüfner and Gausser) ...	—	15,000 to 16,000
,, (Reid)	—	65,000
,, (Roaf) under varying conditions from ...	—	5,000 to 70,000
,, but under more natural conditions and in neutral solution about ...	—	14,000 to 18,000
Histone and Protamine (present communication)	19·3	8,780

The solution complexity accordingly is less than that of the coagulable proteins, but is considerably higher than that of the acidic protein caseinogen, which is by far the lowest of all proteins hitherto examined. The alkalized serum and the histone lie nearly at the same level.

It is interesting to compare the molecular weight in solution as given directly by osmotic pressure with the molecular weights assigned from analysis by Kossel and his co-workers, and with the molecular weights of the amino-acids (hexone bases) which the protamines yield on hydrolysis. The molecular weights given by Kossel for salmine and sturine lie between 800 and 900, so that even after allowance is made for the inorganic acid in the sulphate there must be eight or more such large molecular groups in the solution aggregate to yield 8,000 to 9,000 as found. The mean molecular weight of the amino-acids forming this class of protein may be taken as 150, and, making allowance for the sulphate in combination, this leads to the conclusion that there are about 40 molecules of such amino-acids united to form the complex, or colloidal solution aggregate, of the protamine, or histone, in solution.

At present, similar measurements of the molecular complexity in solution of nucleoproteins and nucleic acids are being carried out.

REFERENCES.

Matthews (1897), *Zeitsch. physiol. Chem.* 23, 399.
Moore, Roaf and Whitley (1905), *Proc. Roy. Soc. B.* 77, 102.
Moore and Roaf (1907), *Biochem. J.* 2, 34.

XIV. THE FATTY ACIDS OF THE HUMAN BRAIN.

By EGERTON CHARLES GREY, 1851 *Exhibition Scholar.*

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In the following communication evidence is brought forward that the solid fatty acids derived from the lipoids of the human brain do not consist chiefly of palmitic and stearic acids as has been claimed by certain investigators. The observation of Thudichum [1901] that the stearic acid of the human brain melts not at 69° but at 52° has been confirmed, and by fractional precipitation an acid isomeric with stearic acid has been isolated.

I have been unable to find any unoxidised fatty acids containing more than eighteen carbon atoms in the molecule. It has also been shown that at least 25 % of the solid fatty acids of the human brain are hydroxy-acids of higher molecular weight than stearic acid. From the close resemblance in properties which the cerebral hydroxy-acids show to the fatty acids found by Darmstaedter and Lifschütz [1896] in lanoline, it may be of some interest to suggest that we have here another indication of the relationship of the nervous system to other tissues of epiblastic origin.

The cause of the high molecular weight of the brain fatty acids.

About two years ago I pointed out [1910] that the mean molecular weight of the solid fatty acids of the human brain, as determined by titration, was considerably higher than that of stearic acid, this fact being confirmed by analysis of the lead soaps. I was not at that time acquainted with Thierfelder's paper on cerebronic acid [1905]. This acid occurs with other hydroxy-acids of high molecular weight in the precipitate of potash soaps difficultly soluble in alcohol, which is obtained when the saponified mixture from the fatty acids is allowed to cool. Moreover although the potassium salts of these hydroxy-acids are sparingly soluble in alcohol, they remain dissolved to a considerable extent in the presence of the other soaps and

subsequently separate amongst the first fractions precipitated as barium or magnesium soaps. The existence of hydroxy-acids in the brain other than cerebronic acid, with the possible exception of hydroxystearic acid [Koch, 1902] has not previously been noted. It is to the presence of these hydroxy-acids, however, that the observed high molecular weights are to be attributed. In the case of certain fractions it was observed that the apparent molecular weight varied enormously when the acid was regained by decomposition of the lead, barium or even the sodium soap by hydrochloric acid, the acid so regained possessing a higher molecular weight than before. The same phenomenon was observed in another way. When certain fractions of solid fatty acids of high molecular weight were converted into lead soaps, and these lead soaps subsequently exhausted with hot benzene or toluene (a process which Fränkel [1909] used to purify the lead soaps of the brain fatty acids), a decomposition sets in with the formation of a white precipitate rich in lead, and a brown coloured solution, which contains not lead soap, nor free fatty acid, but a material practically devoid of acid properties. It seemed clear that an anhydride or lactone formation had taken place in both the cases mentioned. That this view is correct is confirmed by the following fact. If the fatty acids are regained from the potash soaps difficultly soluble in alcohol, taking no precaution to avoid the dehydrating effects of mineral acids, it is found that the acid mixture so obtained is sparingly soluble in alcohol even at the temperature of ebullition, and after repeated exhaustion with hot spirit a portion is left which when dissolved in a mixture of chloroform and alcohol is found by titration to be only faintly acid; but if the same material is saponified the soap will be found completely soluble in hot alcohol and the material regained by decomposition with acetic acid is a true acid substance neutralising a considerable proportion of alcoholic potash. It is to the presence of anhydride or lactone-like derivatives of the hydroxy-acids that the observed variations in mean molecular weights as determined by titrating with potash, are due.

The lactone formation under the conditions described occurs most markedly, not with cerebronic acid, but with the hydroxy-acid melting at 73.5°.

Preparation of the fatty acids from the brain substance.

The brain after removal of connective tissue and washing was heated with 20 % potash solution. After cooling, the solution was acidified with hydrochloric acid and the fatty layer filtered off. This was then saponified with 10 % alcoholic potash for 15–24 hours. The solution was evaporated to

dryness with previous admixture of sodium bicarbonate, and the powdered residue extracted with anhydrous ether till the cholesterol was completely removed. The details of this process are described elsewhere [Grey, 1912]. The fatty acids were now converted into lead soaps and treated with ether, which was done with the aid of the centrifuge. The lead soaps insoluble in ether yielded acids with an iodine value of 27·5 %. From 150 g. of total fatty acids were obtained 67 g. of acids forming lead soaps insoluble in ether. The mean molecular weight of the solid acids was 320 as determined by titration. The iodine absorption is due chiefly to admixture with a solid unsaturated acid described later. The fatty acids from the lead soaps insoluble in ether were now neutralised with potash. The precipitate which separated on cooling is represented by fraction No. 1 in the accompanying table, and consists chiefly of hydroxy-acids. The filtrate was subjected to fractional precipitation by magnesium acetate, and the fractions 2–5 obtained. The substance remaining in solution (fraction 6) consists chiefly of the unsaturated acid melting at 42° to the presence of which the iodine-absorbing power of the solid fatty acids is due, earlier fractions being practically devoid of iodine value. The large loss of material was due to the volatilisation of the acids by steam.

TABLE I. *Showing the separation of 58 g. of solid fatty acid from the human brain.*

No. of fraction	How obtained	Weight	Melting point	Mean mol. weight by potash titre
No. 1	From potash soap insoluble in cold alcohol	14·6	84 –84·5°	420
No. 2	From first precipitate of magnesium soap	6·8	68·5–69°	399
No. 3	2nd ditto	6·5	55·5–56°	324
No. 4	3rd ditto	5·4	57 –57·5°	303
No. 5	4th ditto	8·0	47·2–47·6°	251
No. 6	Residue in solution ...	6·0	40 –41°	——

Loss 10·7

The hydroxy-acids of the human brain.

These were obtained from fraction No. 1 (*vide* Table I) and represent the acids forming potash soaps sparingly soluble in alcohol. They also separate from fractions 2 and 3 during the further purification of these. The potash soap was brought into solution by boiling alcohol and partially precipitated by magnesium acetate. The hydroxy-acid mixture so obtained was further fractionated by cooling the hot alcoholic solution of the acids, by the repetition of which process a fraction was obtained almost

insoluble in alcohol even when hot. This acid when dry was a white powder, and melted at 100–101°. The acid is not cerebronic acid. The main portion of the hydroxy-acids consisted of two other substances which were separated by recrystallisation from acetone and glacial acetic acid. The portion most soluble in acetone melted at 73·5°. The same substance was also obtained from the earlier fractions of magnesium soaps obtained from the potassium soaps soluble in alcohol.

In the course of these investigations the possibility of the formation of hydroxy-acids was considered, but since the chief hydroxy-acids found were not dihydroxy-acids, but monohydroxy-acids, they could not have been artificial products. It is possible however that small quantities of dihydroxy-acids may have resulted from the oxidation of unsaturated acids present. Experiments done with coal gas yielded practically the same iodine value as when no special precautions were taken. Moreover the isolation of acids of great degree of unsaturation and in particular of one containing six double bonds, indicates that oxidation during manipulation does not play any considerable part.

Nature of the hydroxy-acids.

Three distinct hydroxy-acids have been separated, but small quantities of others seem also to be present. The three acids isolated melted at 73·5°, 91° and 100–101° respectively. The melting point of this last corresponds to that of cerebronic acid, the quantity obtained was small, but judging by the result of a single combustion analysis the substance is certainly not cerebronic acid. On the other hand the acid melting at 91° corresponds closely in composition with cerebronic acid as determined by many analyses. The melting point of this substance remained constant after many recrystallisations from acetone and glacial acetic acid. The analyses of these acids are set forth in the accompanying table.

TABLE II.

No.	Melting point	Weight taken	CO_2	H_2O	C %	H %	Empirical formula
1	100–101°	0·1296	0·3395	0·1376	71·44	11·80	$C_{17}H_{34}O_3$ or $C_{22}H_{44}O_4$
2 (a)	91·0°	0·1077	0·2960	0·1193	74·956	12·308	
(b)	,,	0·1034	0·2851	—	75·174	—	
(c)	,,	0·1967	—	0·2270	—	12·530	
(d)	,,	0·1327	0·3660	0·1500	75·221	12·544	
(e)	,,	0·2267	0·6267	0·2567	75·420	12·590	
				Mean	75·193	12·493	$C_{25}H_{50}O_3$
3	73·5°	0·1433	0·3804	0·1730	72·398	12·073	
	,,	0·1323	0·3526	—	72·614	—	
				Mean	72·506	12·073	$C_{29}H_{46}O_5$

10—2

The composition of Thierfelder's cerebronic acid ($C_{25}H_{50}O_3$) is $C = 75\cdot38\,\%$: $H = 12\cdot56$ with which substance No. 2 will be seen to be in good agreement.

Substance No. 1 corresponds to the formula $C_{17}H_{34}O_3$ or $C_{22}H_{44}O_4$. The latter formula would be preferable since according to its melting point and other physical properties it can hardly be a lower member of the cerebronic acid series. In this case the acid would be a dihydroxy-acid and might have resulted by the oxidation of a pre-existing unsaturated acid of the formula $C_{17}H_{32}O_2$. Such an unsaturated acid has not yet been described as occurring in brain lipoids.

The acid melting at $73\cdot5°$ corresponds approximately to the formula $C_{20}H_{40}O_3$ but it cannot be regarded as having been yet satisfactorily examined. It is characterised by the readiness with which it may be transformed into a non-acid lactone-like substance, as already explained.

The unsaturated fatty acids of the human brain.

Since the discovery by Henriques and Hansen [1903] of fatty acids in the brain more unsaturated than oleic acid, no separation of the unsaturated acids seems to have been attempted. The following preliminary separation is therefore described.

About two years ago the author observed that when bromine was added to the ether solution of the unsaturated fatty acids, the temperature being kept at $5°$, a white precipitate separated which was found to contain 78–$79\,\%$ bromine, and therefore corresponded to a fatty acid more unsaturated than any previously described as occurring in lipoids. The amount of such highly unsaturated acid was small, nevertheless the result will be seen to be proof of the existence of unsaturated acids of the nature of clupanodonic acid in the human brain. The bromine separation has also shown the presence of the linoleic and linolenic acid series.

Separation of the unsaturated fatty acids.

An amount of $24\cdot1$ g. of unsaturated acids was dissolved in ether and cooled to $5°$, and bromine added until a definite excess was present; the solution was kept in an ice chest all night, and the precipitate ($0\cdot52$ g.) separated by decantation, and washed in centrifuge tubes with ether until all bromine compounds soluble in ether were removed. An aliquot portion of the ether solution of bromides was evaporated as a check on the amount in solution. From the weight of bromides obtained it is easy to determine the percentage of bromine absorbed by the acids, which was found to be $77\cdot5\,\%$.

The ether was now removed from the main quantity of the soluble bromides, the latter washed free from acetic acid by hot water, and after drying shaken with ligroin of boiling point below 45°. A separation immediately followed, the larger portion being soluble while another portion was apparently insoluble in ligroin, but dissolved readily in chloroform. The quantities found together with the percentage of bromine in the fractions is set forth in the following table.

TABLE III. *The separation of 24·1 g. of unsaturated acids.*

Fraction of fatty acids	Weight of bromide found	Fatty acid which this represents	Bromine per cent.	Percentage of the acid in total un- saturated acids
Clupanodenic acid	0·52 g.	0·13	77·7	0·48
Acids of linoleic and linolenic series	5·64	2·37	58·0	9·83
Oleic acid series	34·23	21·43	37·4	88·92

Properties of the individual fractions of the unsaturated fatty acids.

(1) *Bromine compound insoluble in ether.* To the acid yielding this bromine compound I have given the name *clupanodenic acid* since it is still more unsaturated than clupanodonic acid. Although the quantity used in the analysis was small and the results must be regarded as only indicating the existence of such highly unsaturated acids, nevertheless the substance is quite definite and I have since prepared a gram of the bromide by precipitation of another quantity of unsaturated acids.

The *bromide* is a white powder, which turns a faint straw colour when heated to 120°. With further heating in a small tube it was found to blacken at about 200° and to decompose with further application of heat giving a sublimate and odour of stearic acid. Ignited on platinum foil the substance blackened and melted, burning without leaving any appreciable residue. The bromine compound is practically insoluble in ether, chloroform, and benzene, and only slightly soluble in cold alcohol. In hot alcohol it is more soluble and exhibits the properties of an acid. Titrated with alcoholic potash the molecular weight was found to be over 1000. This number was slightly low on account of the access of carbon dioxide owing to the slowness of the titration, and the titration was moreover carried out on a very small amount of material.

The bromine percentage was determined in two ways.

Data from bromine determinations.

　　(*a*) The substance was heated with nitric acid and silver nitrate.

　　　0·2160 g. of bromo-acid gave 0·3946 AgBr. Br = 77·7 %.

(*b*) The substance was mixed with calcium oxide and ignited as in the method of Piria and Schiff.

$$0.0347 \text{ g. gave } 0.0650 \text{ AgBr.} \quad Br = 79.2 \text{ %.}$$

Calculated for dodecabromostearic acid $Br = 77.9$ %.

(2) *Brominated acids soluble in ether but insoluble in ligroin.* This fraction should contain any acids of the linoleic and linolenic series, and the percentage of bromine found is in agreement with this and points to the mixture consisting of both tetra- and hexa-bromine compounds. It would be premature however in view of the complexity of these lipoid fatty acids, to calculate from the bromine figure the proportions of the bromine compounds of either series of which this mixture may consist. During the process of treament with ligroin it was readily seen that at least two substances were present in the portion insoluble in that fluid for while most of the insoluble material was of a dark colour and of a plastic adhesive nature, a small portion was separated by repeated shaking and decantation which was of a light colour and amorphous. This amorphous material became of a glassy transparent nature when dried at 100° but did not melt at that temperature, whereas the main mass of the bromine compound which was insoluble in ligroin melted below 100°.

0.1556 g. of bromine compound heated with lime gave 0.2094 g. AgBr.

$$Br = 57.4 \text{ %.}$$

Calculated for linoleic tetrabromide $Br = 53.33$ %.

„ „ linolenic hexabromide $Br = 63.33$ %.

(3) *Bromine compounds soluble in ether and also in ligroin.* This fraction is generally regarded as consisting of the dibromide of ordinary oleic acid. But in the present case it was found that the liquid fatty acid was admixed with a solid substance corresponding in composition to oleic acid and likewise forming a dibromide soluble in ether and ligroin. The fatty acid has not yet been examined in detail but it is evident that it occurs to a considerable extent mixed with the unsaturated liquid fatty acids of the brain and is difficult to separate from them. The acid is likewise obtained from the solid fatty acid mixture regained from the lead soaps difficultly soluble in ether, and is found left over in this separation, after fractional precipitation of the saturated acids as magnesium soaps (*vide* fraction 6, Table I).

The total bromides soluble in both ether and ligroin gave on analysis a percentage of bromine corresponding to oleic dibromide.

(*a*) 0.4507 g. gave 0.3880 AgBr. $Br = 36.6$ %.

(*b*) 0.4507 g. gave 0.4050 AgBr. $Br = 38.2$ %.

Calculated for oleic dibromide $Br = 36.2$ %.

Solid unsaturated fatty acid. This acid can only be briefly referred to. Further examination will be needed definitely to establish its nature. The acid was obtained from fraction No. 6 of the original magnesium soap separation, and also by exhaustion of the ether-insoluble lead soaps by hot benzene. It was purified by repeated crystallisation from acetone at a temperature of 5°. The melting point remained practically constant at 42°. The combustion figures show the substance to be of the nature of oleic acid.

(*a*)　0·1185 g. gave 0·1286 H_2O.

(*b*)　0·1323 g. gave 0·1467 H_2O ; 0·3703 CO_2.

	a	*b*	Mean	$C_{18}H_{34}O_2$	$C_{18}H_{36}O_2$
				Calculated for	
C	—	76·34	76·34	76·60	75·66
H	12·10	12·32	12·21	12·06	12·61

The saturated fatty acids of the human brain.

The further separation of these acids obtained from fractions 2–5 of the original magnesium soap precipitation (*vide* Table I) was carried out in the usual way. The fractions were subdivided by precipitation with barium acetate and subsequently converted into silver salts which were submitted to combustion analysis. The fatty acids were regained from the silver salts and the melting points determined. In many cases the acids themselves were submitted to combustion analysis. As already pointed out, no acid of more than eighteen carbon atoms was found. The acids isolated and identified were ordinary stearic, palmitic and myristic acid, and in addition, in quantity quite equal to that of ordinary stearic acid, an isomer of stearic acid melting at 51–52 . This therefore confirms the discovery of Thudichum [1901] that the stearic acid occurring in greatest amount in the brain is not ordinary stearic acid melting at 69 but an isomer melting at 52 to which he gave the name neurostearic acid.

GENERAL CONCLUSIONS.

1. At least 25 % of the solid fatty acids of the human brain are hydroxy-acids, of which three have been isolated. Two of these at least are monohydroxy-acids and cannot therefore have been produced artificially by oxidation of unsaturated fatty acids.

2. The unsaturated acids contain besides oleic, linoleic and linolenic acids an acid still more unsaturated to which the name clupanodonic acid has been

given. It combines with twelve atoms of bromine. Such a compound has not previously been described as occurring in fats or oils.

There is present also a solid unsaturated acid melting at 42°, which is probably an isomer of oleic acid.

3. The saturated fatty acids contain normal stearic and palmitic acids and an isomer of stearic acid melting at 51–52°. The presence of myristic acid has also been confirmed.

4. In the resemblance between the hydroxy-acids of the brain, and those of lanoline, there seems to be additional evidence of the relationship of the nervous tissue to other tissues of epiblastic origin.

In conclusion I would express my thanks especially to Dr H. G. Chapman, at whose suggestion this work was begun, and to Professor T. P. Anderson Stuart, in whose laboratory the investigation was carried out.

REFERENCES.

Darmstaedter and Lifschütz (1896), *Ber.* **29**, 1474; 2890.
Fränkel, S. (1909), *Biochem. Zeitsch.* **21**, 337.
Grey, E. C. (1910), *Proc. Linn. Soc., New South Wales*, **35**, 295.
—— (1912), *Australian Assoc. Adv. Sci.*, **13**, 70.
Henriques and Hansen (1903), *Skan. Arch. Physiol.* **14**, 3.
Koch, W. (1902), *Zeitsch. physiol. Chem.* **36**, 134.
Thierfelder (1905), *Zeitsch. physiol. Chem.* **43**, 215, **44**, 366.
Thudichum (1901), *Die chemische Konstitution des Gehirns der Menschen und der Tiere*, Tübingen.

XV. AN INVESTIGATION OF PHYTIN.

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The chemistry of the substance phytin has attracted the attention of workers in all parts of the world. It is generally regarded as the calcium magnesium salt of inositol phosphoric acid (phytic acid), but the presence of organic constituents other than inositol has been described.

OCCURRENCE.

Phytin occurs in the seeds of a large number of plants and was first extracted from them by Palladin [1895], who distinguished it from coagulable protein. Schulze and Winterstein [1896] prepared it by the same method and showed that it contained phosphoric acid, calcium and magnesium. They supposed it to be identical with the aleurone or globoid bodies described by Pfeffer [1872]. A better method of preparation was discovered by Posternak [1903] and he extracted the substance from various seeds. Hart and Andrews [1903] found that the phosphorus contained in the bran of cereals was chiefly in an organic form of combination, and Patten and Hart [1904] isolated a substance from wheat bran which they regarded as identical with phytin; the same substance was isolated from other cereals by Hart and Tottingham [1909] and Anderson [1912, 4] has prepared it from cotton seed meal. The distribution of phytin in various plants has been investigated by Suzuki and Yoshimura [1907], Staniszkis [1909], and Geys [1910].

PREPARATION.

Palladin and Schulze and Winterstein prepared phytin by extracting the fat-free seeds of *Sinapis nigra* with 10 per cent. sodium chloride solution, boiling to remove coagulable proteins and filtering when the solution had

cooled. On again boiling the solution the phytin was precipitated; it was filtered off, washed with boiling water and dried.

The method of preparation used by Posternak has been patented and phytin is now a commercial article (manufactured by the Gesellschaft für chemische Industrie in Basle). The preparation is as follows.

Oil-cake is extracted with dilute hydrochloric acid (0·5–1 per cent.) and the extract is freed from protein by one of the usual methods. Sufficient sodium acetate is added to remove free hydrochloric acid and then the necessary amount of a soluble calcium salt is added to bring the calcium content of the solution to a fixed amount. The phytin is completely precipitated with copper acetate as a complex copper, calcium and magnesium salt. The precipitate is washed with distilled water and decomposed with hydrogen sulphide. The filtrate from the copper sulphide is evaporated *in vacuo* to a syrup, treated with alcohol and the solid mass dried and powdered.

The extract may be freed from impurities by oxidation, and organic acids such as formic may be used instead of hydrochloric acid.

Phytic acid may be prepared by extracting acidified solutions of phytin with a mixture of one volume of alcohol and three volumes of ether. A syrup is obtained on evaporation which may be precipitated as copper salt as before.

Acid salts of phytic acid may be prepared by dissolving phytin in dilute mineral acids and precipitating with alcohol; with just sufficient acid to dissolve the phytin the precipitate is insoluble in water, but if more acid be used a precipitate of the soluble acid salt of the type CaH_2X is obtained; with still more acid the precipitate is the acid salt of the type CaH_6X.

COMPOSITION OF PHYTIN.

The method of preparation of phytin from extracts of seeds and bran does not exclude the presence of calcium and magnesium phosphate in the preparations which have been made.

There are many conflicting statements concerning the precipitation of phytin by ammonium molybdate; some authors have stated that it is precipitated, some that it is not precipitated; some have said that it gives a white precipitate, others a yellow precipitate. The yellow precipitate has been attributed by many observers to impurities in the phytin. Starkenstein [1910] claimed to have shown that phytin contained inorganic phosphate and that it was decomposed by drying at 100°, but Jegoroff [1912] showed the contrary. Jegoroff pointed out that there is no satisfactory method for

estimating inorganic phosphate in the presence of phytic acid though in some cases that of Schulze and Castoro had been of use. Fingerling and Heeking [1911] have also complained of the absence of a suitable method for the estimation of inorganic phosphate in extracts of fodders containing phytic acid.

None of the investigators mention the methods by which they have ascertained the calcium, the magnesium and the phosphoric acid content of phytin. Most of the methods for the estimation of calcium and magnesium in the presence of phosphoric acid are long and involve possibilities of error. This may account for the very different values which have been found, e.g. :

		Ca	Mg	P	Na	K	Observer
CaMg salt	...	—	—	15·13	—	—	Schulze and Winterstein.
Mg salt ...	...	—	7·78	18·44	—	—	Winterstein.
CaMg salt	...	13·42	8·05	22·30	—	—	Posternak [1900]*.
CaNa salt	...	8·41	—	19·42	18·79	—	,, [1903].
,,	...	8·16	—	19·13	19·02	—	,, ,,
CaMgK salt	(wheat)	1·13	5·80	16·38	—	2·60	Patten and Hart.
,,	(maize)	0·48	6·70	14·17	—	5·60	Hart and Tottingbam.
,,	(oats)	8·60	4·70	16·70	—	1·50	,, ,,
,,	(barley)	0·00	7·90	14·46	—	11·20	,, ,,
,,	(rice)	5·18	17·48	23·48	—	trace	Suzuki and Yoshimura.
CaMg salt (commercial)		11·96	1·45	20·32	—	—	Horner.
CaMgK salt (from phytic acid)		13·03	4·29	19·09	—	6·42	Anderson [1912, 2].
CaMgK salt (wheat bran)		2·82	4·72	14·42	—	—	,, [1912, 3].

The carbon, hydrogen and phosphorus content of phytin and of phytic acid as given by various workers are also very different :

|		C	H	P	Observer
Phytic acid	... ...	9·87	3·70	25·89	Posternak.
,,	... ...	9·97	3·66	26·00	,,
,,	(wheat) ...	10·63	3·38	25·98	Patten and Hart.
,,	(maize) ...	10·30	—	26·07	Hart and Tottingham.
,,	(oats) ...	10·22	—	25·97	,, ,,
,,	(barley) ...	10·54	—	25·88	,, ,,
,,	(cotton seed)	11·71	3·07	26·35	Anderson [1912, 4].
,,	,,	10·89	3·25	27·11	,, ,,
,,	,,	11·02	3·30	26·36	,, ,,
Calculated for $C_6H_{24}O_{27}P_6$		10·08	3·36	26·05	—
CaMg salt	... ...	9·65	2·88	15·13	Schulze and Winterstein.
CaNa salt	... ...	7·25	1·34	19·42	Posternak.
,,	... ...	7·43	1·49	19·13	,,
CaMgK salt (wheat bran)		17·30	3·63	16·38	Patten and Hart.
,,	,,	21·47	3·69	14·42	Anderson [1912, 3].

* Cited in Jegoroff's paper.

The difficulty of burning all the carbon in the presence of phosphoric acid has never been mentioned and the different values may be assigned to this cause.

The Preparation of Phytic Acid.

Phytic acid was prepared by Posternak, who described it as a thick transparent brownish syrup. Patten and Hart again prepared phytic acid from the phytin of the bran of wheat. They pointed out the great difficulty in obtaining it free from calcium (compare the precipitation of phytin with copper acetate) and they adopted the following procedure. The copper compound which is precipitated from the extract is decomposed by hydrogen sulphide. The filtered solution is made alkaline with sodium hydroxide and precipitated with barium chloride. The barium salt is washed until free from alkali, suspended in water and decomposed with the exact amount of sulphuric acid to combine with the barium. The solution is again precipitated with barium chloride in alkaline solution and treated as before. The process is once more repeated and then copper acetate is added. The copper compound which is precipitated is washed, suspended in water and decomposed with hydrogen sulphide. The filtrate from the copper sulphide is evaporated to a syrup and the residue is dried at 110°.

Constitution of Phytic Acid.

The evidence for the constitution at present accepted for phytic acid is based upon the work of Winterstein and of Posternak. Winterstein [1897] prepared a magnesium salt of phytic acid and obtained on hydrolysis with concentrated hydrochloric acid at 130° a 60 per cent. yield of inositol. Posternak analysed the acid and determined its molecular weight by the freezing point method. He gave it the formula $C_2H_8O_9P_2$ and represented it as anhydro-oxymethylene diphosphoric acid,

$$O\begin{cases} CH_2-O-PO\,(OH)_2 \\ CH_2-O-PO\,(OH)_2. \end{cases}$$
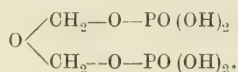
He confirmed the observation of Winterstein that it yielded inositol on hydrolysis (98 per cent. yield) and explained the formation of this product by the polymerisation of the formaldehyde which is first formed.

This constitution of phytic acid was challenged by Neuberg [1908, 1] who quoted Suzuki and Yoshimura's result that inositol is obtained by hydrolysis with the enzyme, phytase. Winterstein [1908] was also of opinion that the inositol nucleus existed in phytic acid. Neuberg and Suzuki and Yoshimura

[1907] put forward formulae representing phytic acid as inositol hexa-phosphoric acid:

$(HO)_3P—O—P(OH)_3$ (Neuberg)

(HO)₂OP—O—HC—CH—O—PO(OH)₂ ... (Suzuki and Yoshimura)
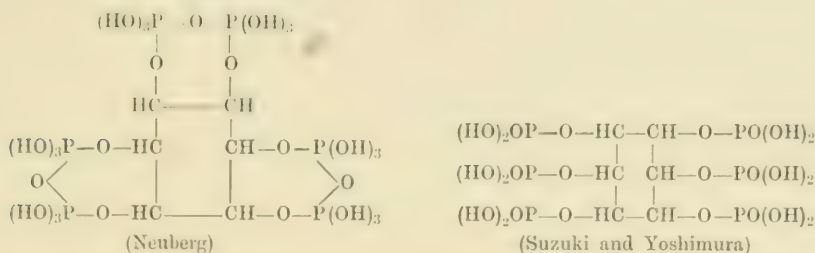
Contardi [1909] contributed further evidence to show that phytic acid is a phosphoric acid ester of inositol: he obtained 18 g. of inositol from 100 g. of phytin by acid hydrolysis.

Starkenstein [1910] from titrations of phytic acid supposed that phytic acid was derived from pyrophosphoric acid and Vorbrodt [1910], who analysed a "sphero" crystalline barium salt, believed that the phytic acid molecule contained two inositol nuclei.

The synthesis of esters of phosphoric acid and inositol has been effected by Contardi [1910] and by Anderson [1912, 1]. Contardi heated together inositol and phosphoric acid and obtained inositol hexaphosphoric acid together with a quantity of inositol diphosphoric acid. The inositol hexaphosphoric acid agreed very closely in its properties with natural phytic acid. By the same method Anderson obtained inositol tetraphosphoric acid as the chief product of the reaction; inositol hexaphosphoric acid was not formed. Anderson [1912, 2] has also prepared an ester of inositol and pyrophosphoric acid. Carré [1911] has maintained that the substances prepared by Contardi are mixtures of inositol and phosphoric acid, but Contardi [1912] refuted these statements.

Most observers seem to agree that phytic acid is composed of only inositol and phosphoric acid, but the data concerning the yield of inositol obtained on hydrolysis do not support this conclusion. Posternak alone obtained a nearly theoretical yield; other workers have obtained yields varying from 20 to 75 per cent. and in the majority of cases the yield has not been given. Levene [1909] claimed to have separated phytin into two constituents; one of them yielded inositol on hydrolysis; the other a carbohydrate which gave pentose reactions and furfural on distillation with hydrochloric acid. Neuberg [1909] severely criticised the claims of Levene and showed that inositol gave small quantities of furfural when distilled with concentrated hydrochloric acid. Considering the scanty data of the yield of

inositol on hydrolysis, Levene's claim that another organic constituent is present in phytin must be considered. Anderson [1912, 3] finds that the phytin of bran does not consist of one substance, but is a mixture.

The above summary of the work on phytin shows that there are several lines of investigation which require attention:

(1)   The presence of inorganic phosphates in phytin.

(2)   The analysis of the calcium and magnesium content of phytin.

(3)   The removal of calcium in the preparation of phytic acid.

(4)   The yield of inositol by the hydrolysis of phytin or phytic acid.

(5)   The presence of other organic compounds besides inositol in phytic acid.

Our investigations are not complete but we believe we have sufficient data to justify their publication, especially as our joint work has ceased for some time, although we hope to continue it at some future date.

Experimental.

Commercial phytin has been employed almost entirely in these investigations, but for comparison phytin (neutral calcium phytate) was prepared from bran. 200 g. of wheat-bran were extracted with successive quantities of N/5 hydrochloric acid until no appreciable amount of phosphorus was contained in the extract. The combined extracts were made alkaline with ammonia; this caused the precipitation as neutral calcium phytate of the whole of the calcium in the extract. The residual phytic acid in the solution was precipitated by adding calcium chloride solution until no further precipitation ensued. The whole was then filtered and the precipitate thoroughly washed with water and dried. The product was dissolved in dilute hydrochloric acid and again thrown down by adding ammonia. The precipitate was re-dissolved in hydrochloric acid and the solution decolourised by shaking with charcoal. After filtering, the phytin was precipitated with ammonia and filtered off. It was washed four times by removing from the filter, triturating with distilled water and filtering, and then dried at 100°. Yield = 6 g. or 3 per cent.

Analysis of Phytin.

(i)   *Estimation of the Total Phosphorus.* The total phosphorus content of phytin was estimated by the method of Neumann as modified by Plimmer and Bayliss [1906]. Several samples were analysed. The analytical data are all given on p. 168.

(ii) *Estimation of Inorganic Phosphorus.* Inorganic phosphate in the presence of organic phosphates can be readily estimated by precipitation with ammonium magnesium citrate as ammonium magnesium phosphate and conversion into magnesium pyrophosphate as was shown by Plimmer and Bayliss in the case of caseinogen. The same method can be applied to the estimation of inorganic phosphate in the presence of glycerophosphate and ethyl phosphate, as was shown by one of us in a previous communication, but in the presence of phytic acid this method cannot be employed. The solution of phytic acid obtained after removal of the calcium by oxalic acid or an oxalate not only inhibits the precipitation of small amounts of inorganic phosphate as ammonium magnesium phosphate but also, when larger amounts are present, renders the precipitation slow and the precipitate consists of ammonium magnesium phosphate and ammonium magnesium phytate. This is shown by the following experiments.

Various amounts of a solution of sodium phosphate were added to a solution of phytic acid prepared by adding oxalic acid to 10 g. of phytin dissolved in water, filtering and diluting to one litre.

A.   10 c.c. phytic acid sol. + 1 c.c. Na_2HPO_4 sol. gave no ppt.

　　..　　　　　　　..　　　　+ another 1 c.c. Na_2HPO_4 sol. gave no ppt.

　　..　　　　　　　..　　　　+ yet another 1 c.c. Na_2HPO_4 gave ppt. = 0·0692 g. P_2O_5.

		Hence ppt. from phytic acid
B.　10 c.c. phytic acid sol. + 1 c.c. Na_2HPO_4 sol. gave ppt. = 0·0313 g. P_2O_5.		= 0·0072
,,　　　　,,　　　+ 2 c.c.　　,,　　　　,,　　= 0·0629　,,		= 0·0147
..　　　　,,　　　+ 3 c.c.　　,,　　　　,,　　= 0·0925　,,		= 0·0202
1 c.c.　　,,　　　　,,　　= 0·0241　..		
C.　10 c.c. phytic acid sol. + 5 c.c. Na_2HPO_4 sol. gave ppt. = 0·1187 g. P_2O_5.		= 0·0075
,,　　　　　,,　　　+ 5 c.c.　　,,　　　　,,　　= 0·1350　,,		= 0·0238
5 c.c.　　,,　　　　,,　　= 0·1112　,,		

The estimation of inorganic phosphate in the presence of phytic acid was carried out by Hart and Andrews [1903] by precipitation with ammonium molybdate at 65° in the presence of very dilute nitric acid. This method was used by McCollum and Hart in their phytase experiments. Under these conditions there is a possibility that phytic acid is hydrolysed with the liberation of inorganic phosphate. Experiments were therefore made to ascertain if inorganic phosphate was completely precipitated by ammonium molybdate in the presence of seminormal to normal nitric acid at room temperature. The yellow solution which is first formed on adding ammonium molybdate to a solution of sodium phosphate in the presence of dilute nitric acid gradually deposits ammonium phosphomolybdate in a crystalline state and the solution becomes colourless in 24 to 48 hours. On determining the

amount of P_2O_5 in this precipitate by the Neumann method, by dissolving it, after filtration and washing, in semi-normal sodium hydrate, boiling off the ammonia and titrating with semi-normal sulphuric acid it was found to be quantitative:

1 c.c. Na_2HPO_4 sol. gave $Mg_2P_2O_7 = 0.0241$ g. P_2O_5.

1 c.c. ,, ,, $+25$ c.c. $H_2O + 25$ c.c. $2N \cdot HNO_3$ $+25$ c.c. Am. molybdate sol. $\Big\}$ gave $\begin{cases} 0.0226 \text{ g. } P_2O_5. \\ 0.0219 \quad ,, \end{cases}$

Applying this method to the solution of phytic acid the following amounts were obtained:

10 c.c. phytic acid sol. $+25$ c.c. $2N \cdot HNO_3 + 25$ c.c. H_2O $+10$ c.c. Am. molybdate sol. $\Big\}$ gave $\begin{cases} 0.0043 \text{ g. } P_2O_5. \\ 0.0042 \quad ,, \\ 0.0044 \quad ,, \end{cases}$

Total P_2O_5 in 10 c.c. phytic acid sol. by Neumann's method $= 0.0871$ gm.

Further proof that the estimation of inorganic phosphate in the presence of phytic acid can thus be effected is given by the following experiments which were carried out in the same way:

(a) 1 c.c. Na_2HPO_4	(b) 10 c.c. phytic acid sol.	(c) 1 c.c. Na_2HPO_4 $+10$ c.c. phytic acid sol.	Sum of (a) and (b)
0.0223 g. P_2O_5	0.0044 g. P_2O_5	0.0264 g. P_2O_5	0.0267
0.0226 ,,	0.0054 ,,	0.0270 ,,	0.0281
0.0222 ,,	0.0051 ,,	0.0270 ,,	0.0273

Hydrolysis of the phytic acid by dilute nitric acid at room temperature therefore does not occur. Employing this method of estimation it has been shown that hydrolysis does not occur at 37° but at 65° and at 75° phytic acid is slowly hydrolysed [Plimmer, 1913, 2, p. 79].

If proteins be present in solution they are precipitated by phosphomolybdic acid with the ammonium phosphomolybdate. The estimation can be carried out by filtering the yellow precipitate, washing free from acid, dissolving in sodium hydrate and then precipitating in the usual way with magnesium citrate mixture and ammonia and finally converting into magnesium pyrophosphate.

The above three solutions after the estimation by Neumann's method were filtered and the inorganic phosphate estimated as magnesium pyrophosphate:

(a)	(b)	(c)	(a) + (b)
0.0239 g. P_2O_5	0.0042 g. P_2O_5	0.0281 g. P_2O_5	0.0281
0.0240 ,,	—	0.0290 ,,	—
0.0239 ,,	0.0050 ,,	0.0289 ,,	0.0289

The estimation of inorganic phosphate in phytin can be readily effected by this method; the presence of calcium does not interfere with the

precipitation. A known weight of phytin is dissolved in normal nitric acid, excess of ammonium molybdate is added and the solution is allowed to stand. When the solution is colourless the crystalline precipitate is filtered off and estimated by the Neumann method, or by filtering, washing free from acid, dissolving in sodium hydrate and precipitating as ammonium magnesium phosphate.

The results of the estimations in different samples of phytin are given on p. 168.

(iii) *Estimation of Calcium and Magnesium.* The previous investigators have not stated the method which they employed for the estimation of these elements in phytin. They have in all probability precipitated the calcium as oxalate and converted it into calcium oxide, either before or after oxidation of the organic matter, and estimated the magnesium in the filtrate as magnesium pyrophosphate. This method involves several possibilities of error; if phosphoric acid be not removed it may be thrown down with the calcium oxalate and some of the magnesium may also be precipitated as magnesium oxalate. The method of separation of these constituents in a mixture is again a long and tedious process.

If oxalic acid or an oxalate be added to a solution of phytin a precipitate is formed, but this precipitate does not consist of pure calcium oxalate. This is shown by the following experiment:

10 g. of commercial phytin were dissolved in decinormal acetic acid and filtered from a small quantity of insoluble matter. This was dried at 110° and analysed: it contained 13·74 per cent. of calcium (estimated as $CaSO_4$, see later) and 17·4 per cent. of phosphorus (by Neumann's method). The filtrate was precipitated with 4·1 g. of oxalic acid dissolved in 50 c.c. of water. The precipitate was washed and dried at 110°; it weighed 5 g. and was analysed:

0·2188 g. heated to redness to constant weight gave 0·0956 g. residue. Calculated amount for pure calcium oxalate is 0·0957 g.

0·3364 g. gave 0·2782 g. $CaSO_4$ (see later) and 0·0031 g. P by Neumann's method.

0·3030 g. gave 0·0028 g. P.

$$Ca = 24·31 \text{ per cent.} \quad (COO)_2Ca \text{ requires } Ca = 31·25 \text{ per cent.}$$
$$P = 0·92 \text{ per cent.}$$
$$P = 0·93 \text{ per cent.}$$

Another preparation of calcium oxalate from phytin contained 0·77 per cent. total P and 0·27 per cent. inorganic P.

Calcium can therefore not be estimated by precipitating with oxalic acid.

Aron [1907] showed that the calcium in milk, urine, etc., could be estimated as calcium sulphate after oxidation of the organic matter by the

Neumann method. When the oxidation is completed the nitric acid is removed as far as possible by diluting with water and again evaporating. The sulphuric acid which remains is then diluted and four volumes of alcohol are added. The calcium sulphate is precipitated and is filtered off, dried and weighed.

Beyond these few estimations of calcium Aron made no other analyses, but he states that other elements might be estimated in the filtrate.

This method therefore required verification and extension for the estimation of magnesium. Analyses of mixtures of calcium chloride, magnesium sulphate and sodium phosphate were consequently made to test the method. The mixtures were evaporated down in a small conical flask with 10 c.c. of sulphuric acid; the acid was diluted with water and precipitated with four to five volumes of alcohol. The calcium sulphate was filtered off on a Gooch crucible, and dried at a red heat. The magnesium was estimated in the filtrate as pyrophosphate after evaporating off the alcohol, oxidising the remainder of the alcohol with nitric acid, diluting with water and making alkaline with ammonia. Excess of phosphoric acid was present in the mixtures to precipitate the whole of the magnesium. The results were:

Calcium		Magnesium	
Taken	Found	Taken	Found
0·1736	0·2540	0·1484	0·1490
0·1736	0·2066	0·0297	0·0310
0·0347	0·0523	0·1484	0·1493
0·1736	0·1885	0·1484	0·1495
0·0264	0·0569	0·0337	0·0332
0·0264	0·0328	0·0337	0·0332
0·0264	0·0588	0·0337	0·0329

Magnesium can thus be correctly estimated but the values for calcium are too high. The high values are due to the presence of sodium in the solution as is shown by experiments with mixtures of calcium chloride and sodium phosphate and calcium chloride and phosphoric acid in various proportions:

Calcium			Calcium		
Taken	Found	In presence of sodium phosphate	Taken	Found	In presence of phosphoric acid
0·0233	0·0285	5 c.c.	0·0233	0·0233	10 c.c.
	0·1038	10 c.c.		0·0233	15 c.c.
	0·1944	15 c.c.		0·0232	20 c.c.
	0·2740	20 c.c.		0·0231	25 c.c.

The presence of sodium salts in the solution thus causes a serious error in the estimation of calcium as calcium sulphate.

If the amount of alcohol used in the precipitation be reduced and if the amount of sodium or potassium in the solution be not greater than 0·1 g. the

estimation of calcium can be accurately effected, especially if not more than one volume of alcohol be used in the precipitation:

3 volumes alcohol		2 volumes alcohol		1 volume alcohol	
Calcium found	Amount of Na present, in g.	Calcium found	Amount of K present, in g.	Calcium found	Amount of K present, in g.
0·0231	0·0	0·0232	0·0	0·0231	0·0
0·0231	0·0	0·0245	0·0394	0·0228	0·0
0·0284	0·1228	0·0281	0·0591	0·0230	0·0197
0·0268	0·0614	0·0383	0·0788	0·0230	0·0394
0·0258	0·0307	0·0237	0·0197	0·0228	0·0591
0·0238	0·0184	0·0238	0·0158	0·0230	0·0788
0·0233	0·0123	0·0237	0·0118	0·0227	0·0788

The amount of calcium taken in all these experiments was 0·0233 g.

Unless the amount of potassium and sodium (if present) in phytin exceed 20 per cent. (0·1 g. in 0·5 g. required for an analysis) the calcium can be accurately estimated by precipitation with one volume of alcohol as calcium sulphate and the magnesium can be estimated in the filtrate as magnesium pyrophosphate. The calcium and magnesium content of various samples of phytin have been estimated by oxidising the organic matter with 10 c.c. sulphuric acid and nitric acid, removing the nitric acid by diluting and evaporating, adding 10-20 c.c. of water and then one volume of alcohol (20-60 c.c.) to precipitate the calcium sulphate. Alcohol was removed from the filtrate by evaporation. On oxidation with nitric acid and on making alkaline with ammonia the magnesium was precipitated as ammonium magnesium phosphate and estimated as pyrophosphate. Excess of P_2O_5 is always present in phytin: the remainder can be estimated by adding magnesia mixture and determining it as pyrophosphate. The sum of the figures obtained then gives the total P_2O_5 content. It has been found to be the same as by the Neumann method. For the data see p. 168.

(iv) *Loss of Weight on Heating and* in vacuo *over Sulphuric Acid.* Phytin loses weight at 110° and *in vacuo* over sulphuric acid. The greater part of the loss takes place during the first heating: the weight continues to decrease very slowly and loss of weight seems to go on indefinitely. One specimen was heated at 110° for 54 hours. The loss in weight under both conditions is about the same, but it takes place more slowly *in vacuo*.

Part of the loss in weight may be due to alcohol; one of the commercial specimens examined gave the iodoform reaction.

The gradual decrease in weight on heating is probably due to a slow conversion into pyrophosphate.

(v) *Estimation of Carbon and Hydrogen.* In the course of many combustions of organic phosphorus compounds the difficulty of burning all the

11—2

carbon has been noticed. Page [1912] has shown that the values for carbon in the case of the hydroxyphosphinic acids were 1–2 per cent. below the true value even after mixing the substance intimately with lead chromate. The values were still lower by the Fritsch-Messinger wet method of combustion.

Previous observers have never mentioned any difficulty in ascertaining the carbon content of phytin or other organic phosphorus compounds nor have they mentioned any modification of the ordinary method which they used.

One of the specimens of phytin was analysed by mixing intimately with the finest copper oxide and it seems improbable that all the carbon was not burnt.

Results of Analyses.

Total Phosphorus.

Commercial phytin :

Sample 1	$0 \cdot 1120$ g. : $37 \cdot 8$ c.c. $\frac{N}{2}$ NaOH.	$18 \cdot 68$ per cent.
2	$0 \cdot 0682$ g. : $24 \cdot 7$ c.c. ,,	$20 \cdot 04$,,
3		$17 \cdot 74$,,
4	$0 \cdot 0579$ g. : $18 \cdot 7$ c.c. ,,	$18 \cdot 57$,,
5	$0 \cdot 0694$ g. : $24 \cdot 1$ c.c. ,,	$19 \cdot 22$,,
6	$0 \cdot 0614$ g. : $21 \cdot 2$ c.c. ,,	$19 \cdot 11$,,
7	$0 \cdot 0544$ g. : $18 \cdot 7$ c.c. ,,	$19 \cdot 76$,,
8	$0 \cdot 0550$ g. : $18 \cdot 5$ c.c. ,,	$18 \cdot 62$,,
Bran (1)	$0 \cdot 6704$ g. : $15 \cdot 8$ c.c. ,,	$1 \cdot 30$,,
Phytin from bran	$0 \cdot 1326$ g. : $0 \cdot 0714$ g. $Mg_2P_2O_7$*	$15 \cdot 00$,,

* Estimated by Mg method after ppt. of $CaSO_4$.

Inorganic Phosphorus.

Commercial phytin :

Sample 2	$0 \cdot 2664$ g. : $4 \cdot 3$ c.c. $\frac{N}{2}$ NaOH.	$0 \cdot 89$ per cent.
4	$0 \cdot 6152$ g. : $10 \cdot 8$ c.c. ,,	$0 \cdot 97$,,
5	$0 \cdot 9642$ g. : $17 \cdot 1$ c.c. ,,	$0 \cdot 98$,,
6	$0 \cdot 6626$ g. : $11 \cdot 7$ c.c. ,,	$0 \cdot 98$,,
7	$0 \cdot 5350$ g. : $9 \cdot 5$ c.c. ,,	$0 \cdot 98$,,
8	$0 \cdot 8726$ g. : $14 \cdot 8$ c.c. ,,	$0 \cdot 94$,,
Phytin from bran	$0 \cdot 3524$ g. : $16 \cdot 7$ c.c. ,,	$2 \cdot 62$,,

Calcium and Magnesium.

					Per cent.	
					Ca	Mg
Commercial phytin :						
Sample 1	$0 \cdot 4552$ g. : $0 \cdot 1984$ g. $CaSO_4$: $0 \cdot 0092$ g. $Mg_2P_2O_7$.				$12 \cdot 82$	$0 \cdot 44$
2	$0 \cdot 5630$ g. : $0 \cdot 2340$ g. ,, : $0 \cdot 0194$ g. ,,				$12 \cdot 2$	$0 \cdot 75$
3	$0 \cdot 4090$ g. : $0 \cdot 1828$ g. ,, : $0 \cdot 0104$ g. ,,				$13 \cdot 15$	$0 \cdot 56$
4	$0 \cdot 5235$ g. : $0 \cdot 2207$ g. ,, : $0 \cdot 0195$ g. ,,				$12 \cdot 40$	$0 \cdot 81$
5	$0 \cdot 4245$ g. : $0 \cdot 1744$ g. ,, : $0 \cdot 0155$ g. ,,				$12 \cdot 10$	$0 \cdot 78$
6	$0 \cdot 4224$ g. : $0 \cdot 1775$ g. ,, : $0 \cdot 0151$ g. ,,				$12 \cdot 36$	$0 \cdot 78$
7	$0 \cdot 4052$ g. : $0 \cdot 1681$ g. ,, : $0 \cdot 0151$ g. ,,				$12 \cdot 20$	$0 \cdot 81$
8	$0 \cdot 4466$ g. : $0 \cdot 1873$ g. ,, : $0 \cdot 0155$ g. ,,				$12 \cdot 34$	$0 \cdot 76$
Phytin from bran	$0 \cdot 1326$ g. : $0 \cdot 0962$ g. ,, : $0 \cdot 0000$ g. ,,				$21 \cdot 12$	$0 \cdot 0$
Bran (1)	$3 \cdot 3430$ g. : $0 \cdot 0150$ g. ,, : $0 \cdot 0786$ g. ,,				$0 \cdot 13$	$0 \cdot 51$
(2)	$4 \cdot 4868$ g. : $0 \cdot 0176$ g. ,, : $0 \cdot 1116$ g. ,,				$0 \cdot 11$	$0 \cdot 54$
(3)	$4 \cdot 2028$ g. : $0 \cdot 0228$ g. ,, : $0 \cdot 1058$ g. ,,				$0 \cdot 15$	$0 \cdot 55$

Loss of Weight (a) *at* 110° *and* (b) *in vacuo over* H_2SO_4.

Commercial phytin :

Sample 3 (a)	5·0229 g. : 0·3425 g.=6·82 per cent.
3 (b)	5·6868 g. : 0·3874 g.=6·81 ,,
4 (a)	0·7376 g. : 0·0544 g.=7·37 ,,
5 (a)	1·7712 g. : 0·1510 g.=8·52 ,,
6 (a)	1·5502 g. : 0·1164 g.=7·51 ,,
7 (a)	0·8472 g. : 0·0628 g.=7·41 ,,
8 (a)	2·1870 g. : 0·1532 g.=7·00 ,,

Carbon and Hydrogen.

Commercial phytin :

Sample 2   0·3052 g. : 0·0815 g. CO_2 : 0·0917 g. H_2O.   C=7·28 %, H=3·33 %.

0·2392 g. : 0·0655 g. ,, : 0·0740 g. ,,   C=7·47 %, H=3·44 %.

If these figures be compared with those of previous workers (p. 159) considerable differences will be noticed. These figures agree most closely with those of Horner for commercial phytin and of Anderson for the preparation made from phytic acid. The total phosphorus content is generally higher and so is the calcium content, but the magnesium content is much lower ; in all the specimens examined magnesium was less than one per cent. Commercial phytin seems to be the calcium salt and the magnesium may be present as impurity.

The carbon content is also lower, but the hydrogen content agrees with the analyses of other workers.

The commercial preparations are very constant in their phosphorus, calcium and magnesium content, but they differ from the preparation from wheat bran, which was the neutral calcium salt of phytic acid and hence contained less phosphorus and more calcium ; the bran preparation contained no magnesium.

Analyses of the phosphorus, calcium and magnesium in wheat bran were made for comparison. It will be observed that bran contains more magnesium than calcium, which is the reverse of phytin.

PREPARATION OF PHYTIC ACID.

Phytic acid was prepared from commercial phytin and from the phytin from bran by the method of Patten and Hart and also by the removal of the calcium as oxalate. Neither method sufficed to remove the calcium entirely, and it was at first thought that some hitherto undescribed product was present.

Preparation 1. 50 g. of commercial phytin (sample 3 above) were

dissolved in a minimum quantity of dilute hydrochloric acid and just sufficient sodium acetate solution was added to render the solution neutral to Congo-red paper. Copper acetate solution was then added until no further precipitation occurred, and the copper salt filtered off by suction. Since it was impossible to wash the precipitate thoroughly on the paper it was removed, made into a uniform cream with distilled water and passed through muslin to ensure the complete breaking up of all lumps. The mass was filtered and the washing procedure repeated twice. (All subsequent washings were effected in this way.) The washed copper phytate precipitate was suspended in water and the mixture treated with hydrogen sulphide until saturated and set aside for twelve hours. The copper sulphide was filtered off and washed. The filtrate and washings were exactly neutralised with sodium hydroxide and excess of barium chloride solution added. The precipitate of barium salt was filtered off and washed twice as described above. It was then suspended in water and the exact quantity of sulphuric acid necessary to combine with the whole of the barium was added, and the barium sulphate filtered off and washed. The solution was neutralised with sodium hydrate and the whole procedure of precipitation with barium chloride and decomposition with sulphuric acid repeated twice. The solution after removal of the barium for the third time was neutralised with caustic soda and then acidified with acetic acid. Copper acetate solution was added and the copper salt so obtained filtered off, washed and decomposed as described above. The filtrate from the copper sulphide was evaporated to a syrup at 100°.

Preparation 2. A specimen of phytic acid was prepared in the same manner from 5 g. of the phytin from bran.

Preparation 3. 100 g. of commercial phytin (sample 4 above) were dissolved as far as possible in decinormal acetic acid. The insoluble residue (amounting to 4 g.) was filtered off and washed. To the filtrate was added the calculated quantity of oxalic acid (37·5 g. allowing for the residue, which contained 11·81 per cent. of calcium) dissolved in water. The calcium oxalate was filtered off and a portion of the filtrate tested with a little more oxalic acid to ascertain if the calcium had been completely removed; no further precipitate was produced. Since calcium oxalate is known to occlude magnesium oxalate to a considerable degree it was thought that the precipitated calcium oxalate would remove at the same time the small quantities of magnesium present. The filtrate was therefore evaporated to a small volume *in vacuo*; a small amount of calcium oxalate separated out and was filtered off. The solution was then concentrated to a syrup at 100°.

The presence of calcium in the syrups of phytic acid.

In the case of all three preparations of phytic acid it was noticed that after drying at 100° an appreciable scum or skin formed on the surface and that when small samples of each specimen were treated with alcohol a small white flocculent precipitate was formed. Each of the preparations was therefore dissolved in a small quantity of water and sufficient alcohol added to precipitate completely the insoluble substance. This was filtered off, washed thoroughly with alcohol, re-dissolved in water and re-precipitated with alcohol. It separated as a white flocculent precipitate which rapidly became stringy and formed a sticky mass on the bottom of the vessel; on standing under alcohol for some time it became quite hard and apparently crystalline. The yields were: (1) 3 g., (2) 1·3 g., (3) 12 g.

On analysis when dried at 110° these precipitates were found to contain phosphorus and calcium, and the third preparation magnesium as well:

Prep. 1   0·2162 g. : 0·0883 g. $CaSO_4$: Ca = 12·01 per cent.
     2   0·2197 g. : 0·1004 g.   ,,   : Ca = 13·45   ,,
     3   0·2586 g. : 0·0654 g.   ,,   : Ca = 7·46   ,,
            0·0357 g. $Mg_2P_2O_7$: Mg = 3·12   ,,

Prep. 1   0·0527 g. : 21·10 c.c. $\frac{N}{2}$ NaOH : P = 20·79 per cent.
     2   0·0227 g. : 8·95 c.c.   ,,   : P = 21·80   ,,
     3   0·0304 g. : 11·80 c.c.   ,,   : P = 21·50   ,,

Another preparation by the oxalic acid method was found to contain 21·1 per cent. of phosphorus, 11·10 per cent. of carbon and 2·98 per cent. of hydrogen.

The alcoholic filtrates were evaporated to syrups at 100°. They agreed in most of their properties with the descriptions of other workers.

When dried to constant weight at 100° and *in vacuo* over sulphuric acid for three months the syrup from Preparation 1 was analysed:

Prep. 1   0·3444 g. : 0·1498 g. CO_2 : 0·1108 g. H_2O.   C = 11·86, H = 3·57 per cent.
            0·0986 g. : 38·0 c.c. $\frac{N}{2}$ NaOH.   P = 21·34 per cent.

$(C_2H_8O_9P_2)_3$ requires C = 10·09 and H = 3·36 per cent.   P = 26·07 per cent.

The carbon and hydrogen figures are higher than those required for phytic acid whilst the phosphorus figure is much lower. It is very probable that some alcohol is retained in the syrup and is not removed by drying; it is also possible that some esterification took place between the alcohol and the phytic acid.

Formation of Inositol by Hydrolysis of Phytic Acid.

The hydrolysis of phytin by dilute nitric acid at the atmospheric pressure and at various temperatures has been investigated by one of us [Plimmer, 1913, 2]; even after seventeen days the separation of the phosphoric acid was not quite complete.

64 g. of phytic acid (the syrup after removal of the crystalline calcium compound in Preparation 3, prepared from 100 g. phytin by treatment with oxalic acid) were dissolved in 250 c.c. of twice normal sulphuric acid and heated in a corked flask in a boiling water bath for 36 hours; about 55 per cent. of the phosphorus was then present as inorganic phosphate; the heating was continued at 75° in a thermostat for 164 hours; the solution now contained 79 per cent. of the phosphorus in an inorganic form. The hydrolysis was proceeded with for another 118 hours after diluting with four volumes of twice normal sulphuric acid. 84 per cent. of the total phosphorus was now present as phosphoric acid. Since the hydrolysis proceeded so slowly it was discontinued. The solution was evaporated to 200 c.c. and an equal volume of alcohol was added, followed by ether until a permanent cloudiness was produced. Inositol crystallised out when the mixture was set aside at 0° for twenty-four hours and was removed by filtration. Two further quantities were obtained on adding ether to the filtrate and keeping at 0°. The total yield amounted to 4·5 g. The alcohol and ether were distilled from the solution and the volume made up to 500 c.c. This solution contained 10·38 g. total phosphorus and 9·11 inorganic phosphorus, i.e. 1·27 g. organic phosphorus or 5·08 g. of phytic acid (25 per cent P). Allowing for the samples removed during hydrolysis the liquid from which the inositol was isolated contained 48 g. of phytic acid, and allowing for the organic phosphorus still present the amount of phytic acid hydrolysed is 42 g. This quantity should yield 10·06 g. of inositol. Since the amount actually obtained was only 4·5 g. it represents less than 50 per cent. of the theoretical.

The inositol was recrystallised from water, alcohol and ether; it melted at 220–222°. Analysis:

$$0·2140 \text{ g. : } 0·2550 \text{ g. } CO_2 : 0·1230 \text{ g. } H_2O : C = 32·5 \text{ per cent., } H = 6·4 \text{ per cent.}$$
$$C_6H_{12}O_6 \text{ requires } \qquad \dots \qquad \dots \qquad C = 40·0 \text{ per cent., } H = 6·7 \text{ per cent.}$$

The figure for carbon is too low. A residue was left in the boat and amounted to 7 per cent.; it contained particles of carbon which it was impossible to oxidise completely after many hours in a current of oxygen.

This combustion again shows the difficulty of burning carbon in the presence of phosphoric acid. The ash was found to contain 52·14 per cent. of P_2O_5.

SUMMARY.

1. Inorganic phosphates in phytin can be readily estimated by precipitation with ammonium molybdate in semi-normal nitric acid at room temperature.

2. The calcium content of phytin cannot be estimated by precipitation as calcium oxalate, but is easily ascertained by precipitation as calcium sulphate.

The magnesium content of phytin can then be estimated as magnesium pyrophosphate.

3. There is great difficulty in removing the calcium from phytin in the preparation of phytic acid.

4. The yield of inositol obtained on the hydrolysis of phytic acid by acids is not quantitative; we are inclined to believe that another organic constituent is present in phytin, and hope to carry out further experiments on the production of inositol by the hydrolysis of phytic acid at some future date.

REFERENCES.

Anderson (1912, 1), *J. Biol. Chem.* **11**, 471.
—— (1912, 2), *J. Biol. Chem.* **12**, 97.
—— (1912, 3), *J. Biol. Chem.* **12**, 447.
—— (1912, 4), *J. Biol. Chem.* **13**, 311.
Aron (1907), *Biochem. Zeitsch.* **4**, 268.
Carré (1911), *Bull. Soc. Chim.* iv, **9**, 195; *Chem. Zentr.* 1911, i, 1196.
Collison (1912), *J. Biol. Chem.* **12**, 65.
Contardi (1909), *Atti. R. Accad. Lincei*, v, **18**, i, 64; *Chem. Zentr.* 1909, i, 1102.
—— (1910), *Atti. R. Accad. Lincei*, v, **19**, i, 23; *Chem. Zentr.* 1910, i, 1032.
—— (1912), *Gazz. chim. Ital.* **42**, i, 408; *Chem. Zentr.* 1912, ii, 186.
Fingerling and Hecking (1911), *Biochem. Zeitsch.* **37**, 452.
Geys (1910), *Zeitsch. ges. Brauwesen*, **33**, 347–349; *Chem. Zentr.* 1910, ii, 982.
Hart and Andrews (1903), *Amer. Chem. J.* **30**, 470.
Hart and Tottingham (1909), *J. Biol. Chem.* **6**, 431.
Horner (1907), *Biochem. Zeitsch.* **2**, 428.
Jegoroff (1912), *Biochem. Zeitsch.* **42**, 432.
Levene (1909), *Biochem. Zeitsch.* **16**, 399.
Neuberg (1908, 1), *Biochem. Zeitsch.* **9**, 557.
—— (1908, 2), *Biochem. Zeitsch.* **9**, 551.
—— (1909), *Biochem. Zeitsch.* **16**, 406.

Page (1912), *J. Chem. Soc.* **101**, 423.

Palladin (1895), *Zeitsch. Biol.* **31**, 199.

Patten and Hart (1904). *Amer. Chem. J.* **31**, 564.

Pfeffer (1872), *Jahrb. wiss. Bot.* **8**, 465.

Plimmer (1913, 1), *Biochem. J.* **7**, 43.

—— (1913, 2), *Biochem. J.* **7**, 72.

Plimmer and Bayliss (1906), *J. Physiol.* **33**, 439.

Posternak (1900), *Rev. gen. Bot.* **12**, 5, 65.

—— (1903), *Compt. rend.* **137**, 202, 337, 439; *Compt. rend. Soc. Biol.* **55**, 1190.

Schulze and Winterstein (1896), *Zeitsch. physiol. Chem.* **22**, 90.

—— (1903), *Zeitsch. physiol. Chem.* **40**, 120.

Staniszkis (1909), *Anzeiger Akad. Wiss. Krakau*, 95; *Chem. Zentr.* 1909, II, 918.

Starkenstein (1910), *Biochem. Zeitsch.* **30**, 56.

Suzuki and Yoshimura (1907), *Bull. Coll. Agric. Tokyo*, **7**, 495.

Vorbrodt (1910), *Anzeiger Akad. Wiss. Krakau*, A, 414; *Chem. Zentr.* 1911, I, 895.

Winterstein (1897), *Ber.* **30**, 2299.

—— (1908), *Zeitsch. physiol. Chem.* **58**, 118.

XVI. ON THE RELATIONS OF THE PHENOLS AND THEIR DERIVATIVES TO PROTEINS. A CONTRIBUTION TO OUR KNOWLEDGE OF THE MECHANISM OF DISINFECTION.

PART II. A COMPARATIVE STUDY OF THE EFFECTS OF VARIOUS FACTORS UPON THE GERMICIDAL AND PROTEIN-PRECIPITATING POWERS OF THE PHENOLS.

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Evidence set forth in a previous communication on this subject [Cooper, 1912, 2] suggested that the germicidal power of the phenols is due, not to a typical chemical action upon the bacterial proteins, as appears to be the case with formaldehyde, but to a de-emulsifying effect upon their colloidal suspensions as evidenced by the precipitation of proteins when a certain phenol concentration is attained.

Since H. Chick [1910] has shown that disinfection by heat is analogous to the heat-coagulation of proteins, it seems that a similarity in mechanism exists between the germicidal actions of heat and of the phenols.

Hardy [1899] pointed out that the heat-coagulation of proteins consists of two processes, namely, (1) The reaction between the protein and hot water (denaturation). (2) The subsequent separation of the altered protein in a particulate form (agglutination). It is not at present known whether the essential stage in the processes of disinfection by heat and the phenols is a denaturation of the bacterial proteins or a destruction of their colloidal suspension.

The experimental work described in this paper has been undertaken with the object of studying more closely the apparent relationship between the bactericidal action of the phenols and their precipitating effects on proteins.

I.   The Influence of Chemical Constitution upon the Germicidal and Protein-Precipitating Action of the Phenols.

The experimental method.   The minimum concentrations in water of various phenols and certain other substances have been determined at which a visible change in the state of aggregation of the protein occurred.   Small alterations in the magnitude of the protein-particles brought about by such concentrations were indicated by a turbidity; larger alterations by a progressively increasing precipitation of the colloid as the concentrations of the phenols rose.

Two proteins were used in the experiments, namely, gelatin and dialysed egg-albumin; the former was employed in the form of small strips and the latter as its colloidal suspension in water.   The effect on the gelatin was to produce a turbidity in the strip immersed in the solution under examination. Throughout the experiments the weight of protein, volume of solution, and temperature (20°) were kept constant.   Observations of the effects of the various solutions upon the proteins were made immediately after admixture and also after standing one hour.   The results tabulated below are based on the observations made after the one hour's standing.

The experimental results.   The protein-precipitating powers of the various substances have been compared with their germicidal activities, which are expressed in the table below in terms of the concentrations required to disinfect *B. typhosus* or *Staphylococcus pyogenes aureus* in 15 minutes under the standard conditions employed by Chick and Martin [1908].   The concentrations have been selected from two previous papers on disinfection (1) by Morgan and Cooper [1912] and (2) by the author [1912, 1], to which papers the numbers 1 and 2 in the following table (p. 177) refer.

The following parallelisms can be deduced from these results.

1.   The germicidal and protein-precipitating powers of phenol are decreased by the introduction of hydroxyl-groups into its benzene nucleus and increased by the introduction of nitro-groups and also by that of a methyl group.

2.   The monohydric phenols are superior to the monohydric alcohols and also to acetone both as germicides and protein-precipitants.

3.   The effects of substitution in the molecule of phenol upon its germicidal and protein-precipitating powers are frequently of somewhat similar orders of magnitude.   An exact correspondence was not expected, firstly, because it is well known that the carbolic acid coefficient of a

substance varies to some extent with the species of micro-organism employed, and, secondly, because bacterial proteins were not used in the determination of protein-precipitating power.

TABLE I.

Substance	Protein-precipitating power Minimum concns. at which precipitation was visible. (Percentages in terms of weight per volume)	Germicidal power Concentrations killing in 15 minutes. (Percentages in terms of weight per volume)	Organism
	Gelatin 0·1 g.	Vol. of liquid 10 c.c.	
Phenol ...	2·5	0·8 [1]	B. typhosus.
Trinitrophenol (Picric acid)	0·2	0·1 [1]	,,
Resorcinol ...	4·0	2·6 [2]	,,
Catechol ...	5·0	1·7 [2]	,,
Quinol ...	5·0	0·75 [2]	,,
Pyrogallol ...	3·0	1·0 [2]	,,
Phloroglucinol	Satd. solns. (5%) had no visible effect	>2·3 [2]	,,
p-Nitrophenol	0·9	0·46 [1]	Staphylococcus py. aur.
m-Nitrophenol	0·6	0·27 [1]	,, ,,
Trinitrophenol (Picric acid)	0·2	0·14 [1]	,, ,,
Ethyl alcohol⎫	50 % and 100 % had	35·0 [1]	,, ,,
Acetone ...⎬	no visible effect	>10·6 [2]	,, ,,
Phenol ...	2·5	1·0 [1]	,, ,,
Egg-albumin ½ c.c. 10 % solution (0·05 g.).		Vol. of solution containing precipitant = 4·5 c.c.	
Phenol ...	1·0	{ 0·8 [1] { 1·0 [1]	B. typhosus. Staphylococcus py. aur.
m-Cresol ...	0·7	{ 0·5 [2] { 0·3 [2]	,, ,, B. typhosus.
Acetone ...	12·0	>10·6 [2]	Staphylococcus py. aur.
Methyl alcohol	20·0	35·0 [1]	,, ,,
Ethyl alcohol	20·0	{35·0 [1] {32·5 [1]	,, ,, B. typhosus.
Propyl alcohol	12·0	14·0 [1]	Staphylococcus py. aur.

II. THE EFFECTS OF VARIOUS SUBSTANCES UPON THE GERMICIDAL AND PROTEIN-PRECIPITATING ACTION OF THE PHENOLS.

The experimental method employed in the investigation of the precipitating action of the phenols was similar to that described in Section I. The various substances were added to the phenol solutions immediately before admixture with the protein. The results recorded in this section are based on observations of the condition of the proteins after one hour's contact with the phenol solutions.

A. Sodium Chloride.

Reichel [1909] showed that the addition of sodium chloride increased both the bactericidal action of phenol and its solubility in proteins. The germicidal power was about doubled by 10 per cent. salt and quadrupled by 20 per cent. salt, and the effects of salt upon the solubility in proteins were of similar orders of magnitude. Smaller amounts of salt had little influence.

These experiments indicated how in the presence of salt a larger amount of phenol became available for disinfection, but did not throw any light on the mechanism of the germicidal action of this disinfectant. It was therefore of interest to investigate the influence of salt upon the protein-precipitating action of phenol.

Gelatin. The minimum concentrations of phenol, which induced a visible precipitation in the presence of varying amounts of sodium chloride, were determined. The results are tabulated below:

TABLE II.

Percentage of salt present (weight per volume)	Minimum phenol concentrations precipitating the protein
0	3 %
5	2·5
12	1·5
19	0·75

Although saturated aqueous solutions of *m*-cresol (1·25 per cent.) had no visible effects upon gelatin, this protein was precipitated by 0·8 per cent. solutions of *m*-cresol in the presence of 9 per cent. salt and even by 0·4 per cent. solutions in the presence of 20 per cent. of salt.

Dialysed egg-albumin. 3·3 per cent. aqueous solutions of phenol only induced a faint turbidity in a sample of egg-albumin, but in the presence of 6 per cent. salt an extensive precipitation occurred. 1 per cent. solutions of phenol in water and in 6 per cent. salt had no visible effect on the albumin suspension; a turbidity however was induced by the same phenol concentration in 15 per cent. salt, and a marked precipitation in 22 per cent. salt solution.

The precipitating action of phenol upon proteins was thus greatly increased by the presence of salt, but was not appreciably affected until high salt concentrations were attained.

A comparison of the results of experiments with gelatin with those obtained by Reichel [1909] indicates that the germicidal power, the solubility in proteins, and the precipitating action of phenol are increased to very similar extents by the presence of salt.

The increased germicidal power thus appears to be due to an increased de-emulsifying action of the phenol upon the bacterial proteins resulting from the availability within the emulsoid particles of a larger amount of phenol.

B. ALCOHOL.

Kronig and Paul [1897] showed that the germicidal action of phenol upon spores was decreased by the presence of alcohol and that solutions of phenol in 98 per cent. alcohol possessed no bactericidal power.

The author [1912, 2] found that the uptake of phenol by a protein was greatly decreased by the presence of alcohol, the protein absorbing for example eight times as much phenol from aqueous solution as from a solution of phenol in 50 per cent. alcohol. These experiments indicated how in the presence of alcohol less phenol became available for disinfection, but they afforded no explanation of the nature of its germicidal action. It was therefore of some importance to investigate the influence of alcohol upon the protein-precipitating action of phenol.

Gelatin in the form of strips was chiefly employed in the experiments, because it was not precipitated by immersion in alcohol of any strength. Although this protein was precipitated by $2\frac{1}{2}$ per cent. aqueous phenol solutions, there was no indication of precipitation by 5 per cent. phenol solutions in 50 per cent. alcohol, and the extent of the precipitation by 6·25 per cent. phenol solutions in 25 per cent. alcohol was much less than that induced by the above aqueous solutions. Furthermore, 10 per cent. solutions of phenol in absolute alcohol had no visible effect upon gelatin. This is of interest when correlated with Kronig and Paul's observation that solutions of phenol in 98 per cent. alcohol possessed no germicidal power.

A sample of dialysed egg-albumin was immediately precipitated by 1·5 per cent. aqueous phenol solutions, but was not visibly affected by the same concentration in the presence of 10 per cent. alcohol.

Although 0·5 per cent. aqueous solutions of picric acid precipitated gelatin, similar concentrations in 30 per cent. alcohol had no visible effect upon this protein.

The inhibiting effect of alcohol upon the germicidal power of phenol thus appears to be due to a decrease in the de-emulsifying action of the latter upon the bacterial proteins, resulting from a diminution in the amount of phenol available within the emulsoid particles.

C. FAT.

Strips of gelatin were immersed in castor-oil, containing varying amounts of phenol. It was found that solutions of phenol in the fat as strong as 10 per cent. (by weight) had no visible precipitating action upon gelatin, although this protein was precipitated by $2\frac{1}{2}$ per cent. aqueous solutions of phenol.

This can be correlated with the fact discovered by Koch that solutions of carbolic acid in oil possess no germicidal power. The greatly diminished protein-precipitating action of phenol in the presence of fat is probably due, as in the case of alcohol, to a decreased uptake of phenol by the protein, because, while phenol is about three times as soluble in gelatin as in water, the partition-coefficient for phenol between fat and water is six.

D. ALKALI.

Kronig and Paul [1897] showed that, while 4 per cent. solutions of phenol in water exerted a strong germicidal action upon anthrax spores, solutions of sodium phenate equivalent to 4 per cent. phenol possessed no germicidal power.

Morgan and Cooper [1912] showed that p-nitrophenol was about five times as powerful a germicide as its potassium salt. The inhibiting action of alkali upon the agglutination of proteins by heat suggested that the decreased germicidal power might be due to the protective effect upon the bacterial protoplasm of the alkali, produced in the hydrolysis of the alkali phenates, against the precipitating action of the phenols simultaneously liberated. Accordingly the effect of alkali on the protein-precipitating action of the phenols has been studied.

Dialysed serum-albumin: Phenol. The albumin was not visibly affected by 0·9 per cent. solutions of phenol, but was precipitated by 1·7 per cent. phenol. The precipitation was not only completely inhibited by N/50 to N/150 concentrations of caustic soda, but even by a concentration as low as N/300.

Gelatin: Phenol. Although gelatin was precipitated by 2·5 per cent. aqueous phenol solutions, this protein was not visibly affected by 4 per cent. phenol in the presence of N/25 caustic soda and was only very slightly precipitated by the same concentration in presence of N/50 caustic soda.

Traces of organic bases, such as ethylamine and ethylenediamine, also inhibited the precipitation of gelatin and egg-albumin by phenol.

Gelatin: p-Nitrophenol. Gelatin was precipitated by 1 per cent. aqueous solutions of *p*-nitrophenol, but was not visibly affected by the same concentration in the presence of N/100 caustic soda, nor even by 1·4 per cent. (N/10) solutions containing N/10 caustic soda.

Not only could alkali inhibit the precipitation of proteins by the phenols, but when present in concentrations varying from N/1 to N/10 it could disperse egg-albumin already precipitated by phenol.

The results indicate that very low concentrations of alkali are sufficient to inhibit the precipitation of proteins by phenol. As alkali combines with the phenols forming salts, which are partially hydrolysed in solution, the inhibiting concentrations must have been even smaller than those stated above.

In view of these results it was of great interest to investigate the influence of alkali upon the germicidal action of phenol in greater detail, and in particular to study the effects of very low alkali concentrations. The experiments consisted in the estimation of the minimum concentrations of phenol required to disinfect in a specified time under standard conditions in the presence of varying amounts of alkali.

The following results were obtained:

TABLE III.

Organism, B. typhosus.

Concentration of caustic soda present	Minimum concentration of phenol required to disinfect in 15 mins. at 20°
0	9·5 in 1000 (N/10)
N/250	,, ,,
N/50	,, ,,
N/25	11 in 1000 (N/8·5)
N/20	,, ,,
N/15	13·5 in 1000 (N/7)

The germicidal powers of the above concentrations of alkali in the absence of phenol were next determined and are compared in the following table with those of solutions containing initially the same alkali concentrations with varying amounts of phenol.

TABLE IV.

Concentration of NaOH			Time required for complete disinfection
N/500 NaOH			> 5½ hours
N/250	,,		> 5½ ,,
N/100	,,		45 minutes
N/50	,,		20 ,,
N/25	,, ·		< 3 ,,
N/15	,,		< 3 ,,
N/10	,,		< 3 ,,
N/25	,,	+ N/10 phenol	> 15 ,,
N/25	,,	+ N/8·5 ,,	15 ,,
N/20	,,	+ N/10 ,,	> 15 ,,
N/20	,,	+ N/8·5 ,,	15 ,,
N/15	,,	+ N/10 ,,	> 15 ,,
N/15	,,	+ N/8·5 ,,	> 15 ,,
N/15	,,	+ N/7 ,,	15 ,,
N/10	,,	+ N/12 ,,	< 15 ,,
N/10	,,	+ N/10 ,,	15 ,,
N/10	,,	+ N/9 ,,	> 15 ,,
N/10	,,	+ N/7 ,,	> 15 ,,

The above results indicate that alkali decreased the germicidal power of phenol when present in moderately low concentration. While, however, the precipitation of serum-albumin by phenol was inhibited by an initial concentration of N/300 caustic soda and that of gelatin by N/50 caustic soda, bactericidal power was not measurably affected until the initial alkali concentration was raised to N/25. The precipitating action of phenol was therefore much more sensitive to the inhibiting effect of alkali than its germicidal action. This discrepancy is not sufficiently explained by the slight acidity of the broth culture, nor by the toxic action of the alkali, since an analysis of the results tabulated above indicates that not only did small concentrations of alkali such as N/250 exert a very feeble germicidal action, but furthermore the bactericidal action of the alkali was greatly decreased by the presence of phenol. Owing to this depreciating effect N/10 solutions of alkali, containing initially N/7 or N/9 concentrations of phenol, possessed a smaller germicidal power than those which initially contained lower phenol concentrations such as N/10 and N/12.

It is possible that a larger amount of alkali is required to preserve the colloidal suspension from the action of the phenol in the case of the bacterial proteins than in the cases of gelatin and albumin.

E. Acids.

Hailer [1910] showed that acids, arranged according to their germicidal power and also according to their efficiencies in increasing the bactericidal power of phenols, were not in the same order. Thus sulphuric acid exceeded oxalic acid in bactericidal power, but was less effective in increasing the germicidal power of phenol. Some of the experimental results described in the previous pages suggested that the discrepancy might be due to differences in the effects of the acids upon the protein-precipitating action of phenol. This, however, seems not to be the case because it was found that, although acids greatly increased the precipitating action of phenol, sulphuric acid was effective in much lower concentration than oxalic acid.

The effects of alcohol and salt upon the precipitating action were shown to be due to a redistribution of phenol between the water and proteins. The greatly increased precipitating action in the presence of acids however is not sufficiently explained in this way, as it was previously shown [Cooper, 1912, 2] that the addition of acid only very slightly increased the solubility of phenol in protein. It is probably due to the formation of an acid salt of the protein, which is more readily precipitated than the protein itself.

Discussion of Results.

The parallelisms deduced from the study of the effects of changes in the chemical constitution of phenol and of the presence of certain substances (e.g. salt, alcohol, fat) upon its germicidal and protein-precipitating powers support the conclusion arrived at in the previous communication, namely, that the germicidal action of phenol is associated with an alteration in the condition of the colloidal suspensions of the bacterial proteins as evidenced by the precipitation of such proteins as gelatin and albumin when certain phenol concentrations are attained.

It has already been pointed out that the heat coagulation of proteins consists of two stages.

1. A reaction between the proteins and hot water (denaturation).

2. A separation of the protein in a particulate form (agglutination).

It is therefore of interest to attempt to characterise the essential process in disinfection by the phenols as either a denaturation of the bacterial proteins or a de-emulsification of their colloidal solution. Chick and Martin [1911 ; 1912] have shown that the process of denaturation by heat

is accelerated by acid and alkali, and retarded by salt, while the subsequent process of agglutination is facilitated by low concentrations of acid and salt, but inhibited by high concentrations and also by alkali.

It was shown in Section II of this paper that the bactericidal power of phenol, its protein-precipitating action and solubility in proteins were increased to equal extents by the addition of salt. The latter thus appears to have no *direct* effect upon the germicidal and precipitating actions of phenol, but to increase them merely by raising the phenol concentration within the emulsoid protein-particles. It is therefore not possible by a study of the effects of salt to correlate these properties of phenol with either the denaturating or agglutinating action of heat upon proteins.

The fact, however, that the germicidal action of phenol is inhibited by low concentrations of alkali, although to a less extent than its precipitating action upon proteins, suggests that the essential process in disinfection by phenol resembles the final phase in the heat-coagulation of proteins and consists in the destruction of the colloidal suspensions of the bacterial proteins.

Since the process of disinfection by hot water was found [Chick, 1910] to be accelerated by alkali as well as by acid, it is possible that the essential stage in this process is the denaturation of the bacterial proteins by the action of the water and not the subsequent precipitation. A study of the effects of salt upon the velocity of disinfection by hot water will throw light on this problem.

Summary.

1. The germicidal and protein-precipitating powers of phenol are similarly affected by the entrance of various chemical groups into its molecule.

(*a*) The introduction of hydroxyl groups decreases and that of nitro-groups and of a methyl group increases the bactericidal and protein-precipitating powers of phenol.

(*b*) The monohydric phenols are superior to the alcohols both as germicides and protein-precipitants.

2. Sodium chloride increases the germicidal and protein-precipitating action of phenol through increasing its solubility in proteins.

3. Alcohol, on the other hand, decreases the germicidal and protein-precipitating action of phenol through diminishing its solubility in proteins. Solutions of phenol in absolute alcohol exert no germicidal action upon spores and have no visible precipitating action on gelatin.

4. Solutions of phenol in fat also possess no germicidal power and do not visibly precipitate gelatin.

5. While the presence of very small amounts of alkali is sufficient to inhibit the precipitating action of phenol upon proteins, only moderately low alkali concentrations can effect a measurable decrease in its germicidal power. The explanation of this discrepancy is not apparent.

6. The precipitating action of phenol is increased by the addition of acids.

Main Conclusion.

From the evidence set forth in the previous communication and in the present paper it is concluded that the absorption of phenols by bacteria is merely the initial stage in the process of disinfection, and that the germicidal action which follows is due, not to a typical chemical union of the phenols with the bacterial protoplasm, as appears to be the case with formaldehyde, but to a de-emulsifying action upon the colloidal suspension of some constituent protein or proteins essential for the vitality of the organisms.

I desire to express my best thanks to Prof. Martin, F.R.S., for much helpful criticism of this work.

REFERENCES.

Chick (1910), *J. Hyg.* **10**, 237.
Chick and Martin (1908), *J. Hyg.* **8**, 654.
——   (1911), *J. Physiol.* **43**, 1.
——   (1912), *J. Physiol.* **45**, 61, 261.
Cooper (1912, 1), *Brit. Med. J.* I. 1234, 1293, 1359.
—— (1912, 2), *Biochem. J.* **6**, 362.
Hailer (1910), *Arbeit. Kais. Gesund.* **33**, 500.
Hardy (1899), *J. Physiol.* **24**, 158.
Kronig and Paul (1897), *Zeitsch. Hyg.* **25**, 1.
Morgan and Cooper (1912), *8th Intern. Congr. App. Chem.* **19**, 243.
Reichel (1909), *Biochem. Zeitsch.* **22**, 149.

XVII. ON THE RELATIONS OF THE PHENOLS AND THEIR DERIVATIVES TO PROTEINS. A CONTRIBUTION TO OUR KNOWLEDGE OF THE MECHANISM OF DISINFECTION.

PART III. THE CHEMICAL ACTION OF QUINONE UPON PROTEINS.

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Blyth and Goodban [1907] found that when pure cresylic acid was exposed to light and air until it had become brown its germicidal power was measurably increased. This was probably due to the formation of derivatives of quinone through the aerial oxidation of the cresols.

Thalhimer and Palmer [1911] however could not detect any increase in germicidal power when phenol was exposed to light and air for some time.

Morgan and Cooper [1912] showed that some of the aromatic amines possessed a higher germicidal power when coloured by long standing than when purified by redistillation. It is probable that the increased germicidal power was due to the production of coloured quinone derivatives, which are known to be formed in the oxidation of aromatic amines.

Thalhimer and Palmer [1911] showed that quinone itself was a very efficient disinfectant and was superior to phenol, cresol, quinol, phenoquinone, and formalin in germicidal power.

The author [1912, 1] confirmed some of the latter results and showed further that quinone was more efficient as a germicide than the aliphatic ketone, acetone.

In previous communications [Cooper, 1912, 2; 1913] evidence was set forth which strongly suggested that the germicidal action of the phenols was due, not to a typical chemical union with the bacterial proteins, but to a de-emulsifying effect upon their colloidal suspensions. In view of the high bactericidal power of quinone it was of great interest to investigate the

relations of this ketone to proteins, in order to compare the mechanism of its germicidal action with that of the phenols and to arrive at a conclusion as to the possibility of a relationship between its germicidal efficiency and chemical reactivity.

Würster [1889] showed that when quinone was added to warm solutions of various amino-acids a red coloration was developed.

Raciborski [1907] showed that quinone gave a red coloration not only with amino-acids, but also with peptone and several proteins (egg-albumin, serum-albumin, fibrin, globulin, legumin, and nuclein). Toluquinone reacted similarly to quinone, and xyloquinone also gave a colour-reaction with proteins and peptone, but not with glycine and alanine. Phenanthraquinone and anthraquinone, on the other hand, gave no colour reactions with proteins. The authors put forward no explanation of the above phenomena.

The Experimental Results.

The investigation described in this paper is divided into three parts.

Part I deals with the relations of quinone to various proteins.

Part II deals with the nature of the chemical action, which quinone was found in Part I to exert upon proteins.

Part III deals with the relation of the chemical reactivity of quinone to its germicidal power.

I. *The relations of quinone to various proteins.*
Gelatin.

When strips of gelatin were immersed in aqueous solutions of quinone (0·1 per cent.) the protein rapidly developed an intense red colour, but retained its transparency. The reaction was irreversible, as the colour was not removed by prolonged boiling with water or absolute alcohol. The red colour was changed to green by the addition of alkali, but was restored by acidification. The altered gelatin was, furthermore, completely insoluble in hot water, and after immersion for 16 hours in quinone solutions the protein was no longer rendered opaque (precipitated) by phenol. Immersion for 30 minutes did not inhibit the precipitation.

Gelatin after immersion in 40 per cent. formalin for 12 hours was also insoluble in hot water and was no longer precipitated by 5 per cent. phenol, although the original gelatin was visibly affected by 2·5 per cent. phenol solutions.

These facts suggest that the quinone was not merely dissolved by the

gelatin, but had reacted chemically with the protein. The colour-reaction did not occur when the gelatin was immersed in solutions of quinone in absolute alcohol, and it only took place to a very small extent in 20 per cent. alcohol. Solutions of quinone in toluene also gave no coloration with gelatin.

Similarly, although Witte's peptone was coloured intensely red when suspended or dissolved in aqueous quinone solutions, no coloration was observed when it was suspended in an alcoholic solution of this substance.

The interpretation of these facts is probably that the quinone is *dissolved* in the gelatin and proteoses before the chemical reaction, so that an efficient external solvent for quinone (such as alcohol) by decreasing the uptake of this substance by the colloids can inhibit the colour-reaction. (See the effect of alcohol upon the distribution of phenol between water and proteins, Cooper [1912, 2].)

Alizarin, like anthraquinone, gave no coloration with proteins. When however gelatin was immersed in strong aqueous solutions of sodium alizarin-sulphonate (Alizarin red, $C_{14}H_5O_2(OH)_2SO_3Na$) it assumed an intense red colour, but, unlike gelatin treated with quinone, it was still readily soluble in hot water and precipitated by 5 per cent. phenol, and the colour was rapidly removed by washing with cold water.

Alizarin and its sulphonic derivative thus differed from quinone in not reacting with proteins, and this inactivity may explain the fact that a saturated solution (0·2 per cent.) of alizarin and a 1 per cent. solution of alizarin-red exerted no measurable germicidal action upon Staphylococcus py. aur.

Caseinogen.

(Merck's Casein—prepared according to Hammarsten.)

When immersed in aqueous solutions of quinone this protein assumed a purple colour, which was not removed by washing with water or boiling alcohol. Unlike the original caseinogen the coloured product (after being washed with water only) was insoluble in N/5 soda. Contact with the alkali, however, turned it green, but the purple colour was restored by acidification. The altered caseinogen was very slowly soluble in hot concentrated hydrochloric acid yielding a brown solution, and thus again differed from the original protein, which quickly dissolved in this acid forming a violet solution.

Egg-albumin.

As stated by Raciborski [1907] dialysed egg-albumin when mixed with aqueous solutions of quinone soon gave an intense red coloration. The

protein was still coagulated by heat and alcohol, red coagula being formed, which were not decolorised by prolonged washing with water and alcohol. The protein was also precipitated from the red solution by saturated ammonium sulphate. The precipitate was a red flocculent substance, readily soluble in water and becoming dark brown on standing. After this colour change the protein was frequently found to have become almost insoluble in water. The coagula obtained from the original red solution by means of heat, alcohol, and phenol remained permanently red.

Horse-Serum.

When aqueous solutions of quinone were added to horse-serum a red coloration rapidly developed. The serum-proteins were still coagulated by heat and precipitated by alcohol at first reversibly and finally irreversibly. The coagula were red and could not be decolorised by prolonged washing with alcohol or water.

By half-saturation of the red solution with ammonium sulphate a red flocculent precipitate was obtained, which, like the egg-albumin precipitated by ammonium sulphate from solutions containing quinone, became dark brown on standing. The precipitate with magnesium sulphate and the alcohol coagulum, on the other hand, resembled the egg-albumin coagula in remaining permanently red, but they soon became brown when immersed in a solution containing ammonium sulphate and quinone. The cause of these colour-changes could not be discovered.

By acidifying with acetic acid the filtrate from the precipitation with half-saturated ammonium sulphate of the serum containing quinone another red precipitate was obtained, corresponding to the albumin fraction from normal serum. The precipitate quickly redissolved on the addition of water.

It was not found possible to crystallize the quinone derivates of serum and egg-albumin by the application of the usual methods.

Witte's Peptone.

The observation of Raciborski [1907] that solutions of Witte's peptone gave a red coloration with quinone was confirmed. Experiments were next carried out with a view to the isolation of the coloured products.

Witte's peptone has been separated into five constituents by the method of fractional precipitation with alcohol and salt described by Haslam. The following fractions of proteoses have been isolated in this way:

1. Insoluble in 50 per cent. alcohol and water. Crude hetero-proteose.
2. Insoluble in 50 per cent. alcohol and soluble in water.

- (1)   a-proto-proteoses—precipitated by half-saturated $(NH_4)_2SO_4$.
- (2)   a-deutero-proteoses—precipitated by saturated $(NH_4)_2SO_4$.
3.   Soluble in 50 per cent. alcohol and soluble in water.
- (1)   β-proto-proteoses—precipitated by half-saturated $(NH_4)_2SO_4$.
- (2)   β-deutero-proteoses—precipitated by saturated $(NH_4)_2SO_4$.

By similarly fractionating the coloured liquid obtained by mixing solutions of quinone and Witte's peptone together it was possible to isolate corresponding precipitates all of which were highly coloured. Some of these products, however, did not differ merely in colour from the fractionated proteoses. Thus, while the alcoholic precipitate (a-proteoses and hetero-proteoses) obtained from an aqueous solution of Witte's peptone was readily soluble in warm anhydrous m-cresol, the corresponding fraction obtained from the aqueous solution after treatment with quinone was only slightly soluble.

Again, the a-proteoses after reacting with quinone were no longer precipitated by formaldehyde, although they were still precipitated by alcohol, mercuric chloride, and phosphotungstic acid. The significance of this difference is discussed later. The fractions obtained from Witte's peptone after treatment with quinone corresponding to the a- and β- proteoses gave the characteristic test for proteoses, namely a precipitate with nitric acid, soluble on warming and reappearing on cooling.

The main conclusion to be drawn from these results is that proteins isolated after treatment with quinone are permanently coloured and frequently changed in solubility and precipitability, and thus appear to be chemically altered. In the following pages the nature of this chemical change and the possibility of its relationship to the toxic action of quinone upon bacteria are discussed.

II.   *The nature of the chemical action of quinone upon proteins.*

(i)   *The colour reactions given by quinone with simple amines.*

A large number of amines readily gave colorations with quinone in aqueous solution at ordinary temperatures.

(ii)   *The colour-reactions with imino-compounds.*

In cold aqueous solution quinone gave red colorations with methylaniline and di-amylamine, but not even on warming with succinimide, acetanilide, and uric acid. Under certain conditions quinone could thus react with substances containing the $=NH$ group.
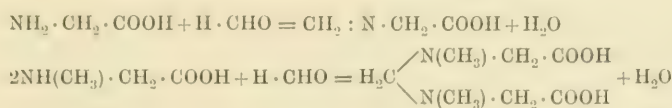
(iii)   *The inhibitory effect of formaldehyde upon the colour-reactions.*

The fact that quinone gives colorations with amino- and imino- compounds

suggests that the colour-reaction with proteins is due to a condensation of the ketonic groups of the quinone with their $-NH_2$ and $=NH$ groups.

Formaldehyde is known to react with amino- and imino- compounds by condensation with the $-NH_2$ and $=NH$ groups, forming methylene derivatives.

$$NH_2 \cdot CH_2 \cdot COOH + H \cdot CHO = CH_2 : N \cdot CH_2 \cdot COOH + H_2O$$

$$2NH(CH_3) \cdot CH_2 \cdot COOH + H \cdot CHO = H_2C \begin{cases} N(CH_3) \cdot CH_2 \cdot COOH \\ N(CH_3) \cdot CH_2 \cdot COOH \end{cases} + H_2O$$

The colour reactions given by quinone with proteins, if due entirely to a similar condensation, should therefore be inhibited by the previous formalisation of these substances.

Substance	Nature of reaction
Ammonia	Brown coloration.
Ammonium sulphate ...	Purple ,,
Ethylamine	Violet ,,
Di-amylamine	Rose red ,,
Guanidine (carbonate) ...	Intense green coloration. (Hot solutions brown.)
Glucosamine	Red coloration.
Amino-antipyrine	Purple ,,
Tryptophane	Red ,,
Atoxyl	Red ,,
Aniline ... ...} o-Phenylene-diamine ...}	Red ,, (followed by brown precipitate).
m-Tolylene-diamine ...	Violet ,, ,, ,, ,,
β-Naphthylamine	Brown ,,
Tetra-hydro-β-naphthylamine	Brown oil.
Pyridine	Dark yellow coloration passing to red.
Succinanilamine	Red coloration (followed by red precipitate).

It was actually found that in the case of egg-albumin, serum-proteins proteoses, glycocoll, glycyl-l-tyrosine, methylaniline, and di-amylamine the colour reactions with quinone were entirely inhibited by adding 40 per cent. formalin to the compounds either previously to or simultaneously with the addition of the quinone. Smaller amounts of formaldehyde (one to ten per cent.) added with the quinone were found to inhibit the colour-reaction partially.

Conversely, the a-proteoses present in Witte's peptone after treatment with quinone were no longer precipitated by formaldehyde, although they were still precipitable by alcohol, phosphotungstic acid, and mercuric chloride.

In the case of gelatin, however, formalisation did not inhibit the colour-reaction with quinone. This was also true in the case of ammonia, and here the absence of any inhibiting effect could not be due to incomplete interaction

with formaldehyde, because the product of this reaction—hexamethylene-tetramine (urotropin), which was proved to be ammonia-free, readily gave a red coloration with quinone.

The purified product from the interaction of formaldehyde and aniline also gave a colour-reaction with quinone.

The inhibiting effect of formalin upon the colour-reaction given by quinone with certain proteins and other amino- and imino- compounds, and its inability to precipitate the compounds of quinone with α-proteoses, although it readily precipitates the proteoses themselves, confirm the view that it is the $-NH_2$ and $=NH$ groups present in the proteins and their hydrolytic products that react with the quinone. It is difficult to understand, however, why the products of formalisation of gelatin, ammonia, and aniline should behave exceptionally in yielding a coloration with quinone.

In order to attempt to understand the mechanism of the interaction of quinone and proteins it is necessary here to set forth the course of the reaction known to occur between an amine, such as aniline, and the above ketone.

The products of the reaction depend upon the experimental conditions, as indicated below.

1. As a result of the action of aniline upon an alcoholic solution of quinone there are three products:

1.   2.   3.

Quinone.   Di-anilido-quinone.   Di-anilido-quinone-anil.   Di-anilido-quinone-di-anil.
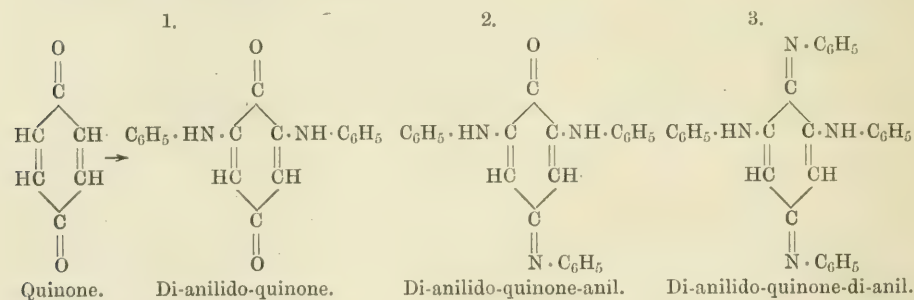
2. In the presence of acetic acid the chief product is di-anilido-quinone-anil.

3. By fusing quinone with aniline and its hydrochloride the chief product is di-anilido-quinone-di-anil.

There are thus two possible reactions between quinone and proteins and their hydrolytic products:

1. The condensation of the ketonic oxygen atoms with the hydrogen of the amino- and imino-groups, forming compounds of the anil-type (see above 2, 3).

2. The replacement of hydrogen attached to the benzene nucleus of quinone by amino-acid residues through the amino- or imino-groups forming compounds of the anilido-type (see above 1, 2, 3).

These two reactions might proceed simultaneously forming compounds analogous to di-anilido-quinone-di-anil.

(iv) *Experiments with quinone-dioxime.*

Attempts were next made to discover to which type of chemical reaction the red colorations given by quinone with proteins were due.

It was thought that experiments with quinone-dioxime could decide this question, because, although substitution in the ketonic groups naturally prevents their condensation with amino- and imino-compounds, it is known not to inhibit the entrance of these substances through their nitrogen atoms into other parts of the quinone nucleus.

Quinone-dioxime was prepared by the action of hydroxylamine hydrochloride upon quinone, the reaction being carried out in acid solution to prevent the reduction of the quinone to quinol.

It was found that aqueous solutions of quinone-dioxime gave no colorations with serum, gelatin, proteoses and alanine. The gelatin after immersion in the quinone-dioxime solution was found to be still soluble in hot water and precipitated by phenol as a white substance, and was thus not chemically altered.

These results strongly suggest that the colour-reaction given by proteins with quinone is due to the condensation of the $-NH_2$ or $=NH$ groups with the ketonic groups of the quinone, compounds similar to quinone-dianil being produced. This conclusion is supported by the fact that no oxime could be obtained by the treatment of the quinone-proteose compounds with hydroxylamine hydrochloride.

The chemical action of quinone upon proteins thus resembles that of formaldehyde.

(v) *The relations of acetone to proteins.*

Since it was found that quinone possessed a germicidal power more than 100 times as great as that of acetone [Cooper, 1912, 1] it was of interest to compare the effects of these two ketones on proteins.

Acetone was found to differ from quinone in exerting a precipitating action upon proteins, but while 0·1 per cent. solutions of quinone gave a colour-reaction with egg-albumin and gelatin, the albumin was not precipitated by aqueous solutions of acetone below 12 per cent. and gelatin was not even affected by immersion in 50 per cent. and 90 per cent. solutions.

The gelatin was still soluble in hot water and precipitable by phenol after this treatment, so that there was no evidence that it was chemically altered by the acetone. This ketone is therefore to be classed with the alcohols and phenols as a protein-precipitant.

III. *The relation of the chemical reactivity of quinone towards proteins to its germicidal power.*

The fact that as a germicide quinone is greatly superior to many other para-di-substitution products of benzene is seen from the following table [Cooper, 1912, 1 and Morgan and Cooper, 1912].

Substance	Organism	Carbolic coefficient
Quinone	*Staphylococcus py. aur.*	10
Benzaldehyde ...	*B. typhosus*	10
Quinol	,,	1
p Cresol	*Staphylococcus py. aur.*	2·4
,, ...	*B. typhosus*	2·6
Aniline	*Staphylococcus py. aur.*	0·5
,, ...	*B. typhosus*	0·57
p-Toluidine ...	,,	1·25
p-Phenylene-diamine	,,	Under 0·3
p-Nitrophenol ...	*Staphylococcus py. aur.*	2·3

This itself suggests that the high germicidal power of quinone is associated with its chemical reactivity.

This conclusion is supported by certain other facts.

1. Not only is quinone superior to phenol, *p*-cresol, quinol, *p*-nitrophenol and acetone in germicidal power, but it can exert a chemical action upon proteins in concentrations (e.g. 0·1 per cent.) much lower than those in which the above substances can induce protein precipitation [Cooper, 1912, 2; 1913].

2. Benzaldehyde, which resembles quinone in its chemical action upon proteins, is also approximately equal to quinone in germicidal power, its carbolic coefficient with *B. typhosus* being 10.

There is thus some evidence that the mechanism of the germicidal action of quinone consists in a chemical interaction with some constituent protein or proteins of the bacteria essential for vitality, and not, as seems to be the case with the phenols, in a precipitating effect upon the colloidal suspension. The superiority of quinone as a germicide to various phenols and to acetone is sufficiently explained by the fact that it reacts with proteins in concentrations much lower than those in which the phenols and acetone exert a precipitating action.

Summary.

1.   The observations of Würster and Raciborski that quinone solutions gave a red coloration with various proteins and amino-acids have been confirmed.

2.   The proteins (egg-albumin, proteins of horse serum, gelatin, Witte's peptone) could be isolated in the coloured condition from the red solutions by means of various precipitants and could not be decolorised by prolonged washing with water or alcohol. Other physical properties of the proteins, e.g. solubility, precipitability, were frequently changed as a result of the treatment with quinone, as is also the case when proteins react with formaldehyde. From these results it appeared that the proteins had become chemically altered by the quinone.

3.   The colour-reaction did not occur when gelatin and proteoses were immersed in solutions of quinone in absolute alcohol. It would appear that the quinone was dissolved by the colloids before the chemical reaction, so that an efficient solvent for this ketone such as alcohol, by decreasing the uptake, could inhibit the colour-reaction.

4.   The addition of sufficient formaldehyde to proteins, proteoses, amino-acids, and imino-compounds either before or simultaneously with the addition of the quinone completely inhibited the colour-reactions. Smaller amounts of formalin decreased the intensity of the red colorations. Gelatin, aniline, and ammonia however behaved exceptionally inasmuch as they still gave the colorations with the quinone after the addition of formalin. The positive results appeared not to be due to incomplete formalisation, since the isolated compounds of aniline and ammonia with formalin gave colour-reactions with quinone.

The inhibitory effect of formalin upon the colour-reactions given by quinone with certain proteins, with proteoses and amino-acids indicates that the latter react with quinone through their $-NH_2$ or $=NH$ groups.

5.   Proteins, proteoses, and alanine gave no colour-reaction with quinone-dioxime, and no oxime could be prepared from the quinone-proteose compounds. This is presumptive evidence that the constituent $-NH_2$ or $=NH$ groups of the proteins and their hydrolytic products condense with the ketonic groups of the quinone. The chemical action of the latter upon proteins thus resembles that of formaldehyde.

6.   Acetone differed from quinone in acting as a protein-precipitant.

7.   There is some evidence that the germicidal power of quinone is due

to its chemical action upon some constituent protein or proteins of the bacterium essential for vitality and that the superiority of quinone as a germicide to phenol, quinol, and acetone is explained by its reactivity towards proteins in much lower concentration.

I desire to express my best thanks to Prof. Martin, F.R.S., for helpful criticisms of this work.

REFERENCES.

Blyth and Goodban (1907), *The Analyst*, May.
Cooper (1912, 1), *British Medical J.* **1**, 1234, 1293, 1359.
—— (1912, 2), *Biochem. J.* **6**, 362.
—— (1913), *Biochem. J.* **7**, 175.
Morgan and Cooper (1912), *8th Internat. Congr. Applied Chem.* **19**, 243.
Raciborski (1907), *Chem. Zentr.* i. 1595.
Thalhimer and Palmer (1911), *J. Infect. Diseases*, **9**, 172.
Würster (1889), *Chem. Zentr.* i. 392.

XVIII. THE RATE OF FERMENTATION BY GROWING YEAST CELLS.

By ARTHUR SLATOR.

If a sugar solution which also contains the necessary food for yeast growth is seeded with a small quantity of yeast, the yeast grows and the sugar is fermented to alcohol and carbon dioxide. If the seeding is small and all necessary food for yeast growth is in excess the growth during the earlier stages of the reaction is unrestricted and follows the logarithmic law of increase, that is the rate of increase is always proportional to the quantity present.

At a later period retarding influences come into action, the yeast multiplication becomes restricted and during the final stages of the fermentation ceases entirely.

In this communication an account is given of some measurements made during the earlier part of the reaction where the yeast growth is unrestricted.

If certain simple assumptions regarding fermentation are made, it can be shown that during this period not only does yeast growth, but also the fermentation caused by the yeast, follow the logarithmic law.

If the medium is seeded with N cells per c.c. then the rate of growth at any time t is proportional to the number of cells present $N + n$, where n is the increase during the time t, that is,

$$\frac{dN}{dt} = K(N + n) \quad \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots(1).$$

where K is the constant of growth.

On integration this becomes

$$K = \frac{1}{t} ln \frac{N+n}{N} \quad \dots\dots\dots\dots\dots\dots\dots\dots\dots(2),$$

or

$$n = N(e^{Kt} - 1) \quad \dots\dots\dots\dots\dots\dots\dots\dots\dots(2\,a).$$

If F represents the number of grams of sugar fermented per unit of time by each yeast cell and s the total amount fermented in time t, then s is determined by the equation

$$s = \int_0^t (N + n) F \, dt.$$

Substituting from equation 2 a we have

$$s = \int_0^t NF\,e^{Kt}\,dt$$

which on integration becomes

$$s = \frac{NF}{K}\,(e^{Kt} - 1)\ \dots\dots\dots\dots\dots\dots\dots\dots\dots(3),$$

or from equation 2 a

$$s = \frac{nF}{K}\ \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots(4).$$

If S is the amount of sugar fermented when the yeast grows from a very small seeding to N, then from equation 4

$$S = \frac{NF}{K}\ \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots(5).$$

Equation 3 therefore becomes

$$s = S\,(e^{Kt} - 1)\ \dots\dots\dots\dots\dots\dots\dots\dots\dots(6)$$

or

$$K = \frac{1}{t}\,ln\,\frac{S+s}{S}\ \dots\dots\dots\dots\dots\dots\dots\dots\dots(6\,a).$$

It is evident from this equation that the time-fermentation curve during this period is logarithmic and that the constant of the curve is the constant of growth.

The validity of these equations has been tested by measuring the rate of growth of a pure culture of a Burton yeast in lightly hopped wort of specific gravity 1·040. It was found that K the constant of unrestricted growth of this culture could be determined by methods of yeast counting and also by measuring the rate of fermentation of the growing yeast cells.

Four different methods of estimating K have been worked out and when tested with this growth of yeast were found to give almost identical results.

In all these experiments the seeding was made with actively growing yeast cells so that any initial retardation in the growth which would take place if older yeast cells were used is eliminated.

METHOD 1.

The medium is seeded with a known number of yeast cells under sterile conditions. Tubes containing the inoculated solution are kept slowly agitated in a thermostat. At certain intervals of time the tubes are taken out and the number of yeast cells counted. K is calculated according to equation 2 which can be put in the form

$$0\text{·}434\,K = \frac{1}{t}\,\log\frac{N+n}{N}.$$

TABLE I.

Temp. 20°.

t = time in hours.

$N+n$ = number of yeast cells per c.c. at time t.

t	$N+n$	$0.434 K$
0	90,100	—
17·3	4,660,000	0·100
0	255,000	—
7·0	1,390,000	0·105
9·0	2,200,000	0·104
0	1,360	—
35	3,550,000	0·098
	Average	0·102 $T = 2.95$ hrs.

In the above table T is the time for the yeast to increase to twice the original amount and is determined by the equation

$$T = \frac{\log 2}{0.434 K} = \frac{0.301}{0.434 K}.$$

METHOD 2.

Measurements of the rate of fermentation are carried out in the manner described in previous publications. [Slator, 1906, 1908.]

The sterile medium (75 c.c.) is seeded with a small seeding of yeast in a tube of capacity about 100 c.c. The tube is connected with a manometer to measure the rate of production of carbon dioxide. With a suitable seeding appreciable fermentation starts in about 15 hours, the reaction proceeds faster as the time goes on and measurements are continually taken on the manometer at suitable intervals.

The readings on the scale can be used directly to test equation 6 a

$$0.434 K = \frac{1}{t} \log \frac{S+s}{S}.$$

TABLE II.

Temp. = 20°.

t = time in hours from first reading S.

$S+s$ = manometer reading in cm., at time t taking the infinitely early reading as zero.

t	$S+s$	$0.434 K$
−15	(0·3)*	—
0	9·7 (S)	—
0·5	10·9	0·101
0·83	11·85	0·105
1·25	12·9	0·099
1·58	13·9	0·099
1·92	15·05	0·099
	Average =	0·101

A second experiment gave $0.434 K = 0.100$. $T = 3.0$ hrs.

* NOTE. 15 hrs. before the first reading is too short a time to give exactly the infinitely early reading and a small correction has to be made to obtain this value. As T is approximately 3 hrs. this correction is easily shown to be 1/32 of S, that is in this case 0·3 cm.

METHOD 3.

The constant of growth can be estimated by means of equation 5

$$K = \frac{FN}{S}.$$

The yeast crop and the amount of fermentation are measured after a convenient amount of fermentation has taken place, the initial seeding of yeast being small. F is determined by a separate experiment. The method is not of much practical value as both N and F have to be determined whilst t the time, a factor most easily measured, is eliminated.

With the culture of yeast growing in wort at 20° it was found that 10^6 yeast cells per c.c. caused a fermentation of 0·620 cm. per hour. In another experiment with growing yeast cells it was found that when the yeast crop was $5·2 \times 10^6$ cells per c.c. the fermentation was 14·4 cm. That is

$$F = 0·620 \times 10^{-6}, \quad N = 5·2 \times 10^6, \quad S = 14·4.$$

It follows therefore that

$$0·434\, K = 0·434\, FN/S = 0·097$$

a result in agreement with the previous experiments.

METHOD 4.

The fourth method involves measurements of the rate of fermentation but has an advantage over method 2, as it allows measurements to be taken in any part of the reaction even where the yeast growth is retarded and nevertheless gives true values of K the constant of unrestricted growth.

The method is as follows: two experiments are made, the medium in each case being seeded with small seedings in a known ratio. If the seedings are small enough the time-fermentation curves are identical except that the one reaction is a definite time behind the other. This time-difference is the time for the one seeding to grow to the other, and from the two values K is easily determined.

If R is the ratio of the two seedings and t_1 and t_2 the times at which the two fermentations reach some definite stage in the reaction, then

$$0·434\, K = \frac{1}{t_2 - t_1} \log R.$$

This method was tested in the following manner. Two flasks of wort were seeded with actively growing yeast, one 319 times the amount of the other. 75 c.c. of the wort with the greater seeding were placed in the tube of the apparatus and the manometer connected. Appreciable fermentation

started in 15 hours and several readings were taken at definite times. The first solution was then replaced by the second which in the meantime had been resting in the thermostat. On the next day the times were taken when the manometer showed the same changes in pressure as in the previous experiment. The various differences of time were in good agreement and the value of $0.434 K$ calculated from the average came to 0.106.

TABLE III.

t_1 = time at which the first reaction gives the reading M on the manometer scale.
t_2 = time on the next day when the second reaction reaches the same point.
Temp. = $20°$. $R = 319$.

M	t_1	t_2	$t_2 - t_1$
9·4	9·20 a.m.	9·0 a.m.	23 hrs. 40 mins.
10·6	9·50	9·32	23 ,, 42 ,,
11·55	10·10	9·52	23 ,, 42 ,,
12·6	10·35	10·18	23 ,, 43 ,,
13·6	10·55	10·38	23 ,, 43 ,,

Average   23 hrs. 42 mins. = 23·7 hrs.

$$0.434 K = \frac{\log 319}{23.7} = 0.106. \quad T = 2.85 \text{ hrs.}$$

A comparison of the values of K determined by the different methods shows good agreement.

TABLE IV.

Method 1 gives $0.434 K = 0.102$
,,   2 ,, $0.434 K = 0.101$
,,   3 ,, $0.434 K = 0.097$
,,   4 ,, $0.434 K = 0.106$

It is evident that the rate of growth of the yeast during this period is regular and can be measured accurately by yeast counting or by fermentation. It is of interest to note that the yeast crop at any time is composed mainly of the last few generations of yeast and any dying off of the old yeast cells would hardly affect the value of K. Further if the yeast crop were composed of cells of different activity correct values of K would be obtained by method 2 if the average value of the fermentative power remains constant during the time of measurement, for equation 6 a involves only ratios of S and s.

The equations and experiments cover only about 2 per cent. of the reaction corresponding to a yeast crop of 10 million cells per c.c., which is about 8 per cent. of the final crop. Growth after this time is measurably retarded, probably mainly by the carbon dioxide which is known to have a considerable retarding influence on yeast growth.

An accurate knowledge of the yeast crop and the fermentation during this early period is however given by these experiments and may be put in the following form.

TABLE V.

Temp. = 20°.
$F = 1.2 \times 10^{-14}$ grms. per sec. $= 4.3 \times 10^{-11}$ grms. per hour. $0.434\,K = 0.100$.

Hrs.	Cells per c.c.	G. per c.c. fermented
—	1,000	1.8×10^{-7}
10	10,000	1.8×10^{-6}
20	100,000	1.8×10^{-5}
30	1,000,000	1.8×10^{-4}
40	10,000,000	1.8×10^{-3}

These equations and methods of estimating the constant of growth can be applied to other micro-organisms. Methods of counting may not always be suitable, but method 4 is probably of general application. The rate of growth of bacteria which produce acid, for instance, could be determined by following the reaction by titration or by electrical conductivity and methods for other growths could be devised without much difficulty.

One of the principal uses of K is to determine the time between infection and the beginning of the chemical action brought about by the organism. The factors which influence K are the factors which determine this time and in extreme cases say whether a liquid is susceptible to the growth of the micro-organism or not.

The value of K may not remain constant over the period of growth in which one is specially interested but a row of constants is not the aim of the investigation and a consistent though variable K may lead to more important results than a constant one.

Micro-organisms grow not only in liquids but also on solids and in the liquid film covering solids and it is of importance to know whether these equations and methods are applicable to determine rates of growth in such cases.

Some information on the subject was obtained by measuring the rate of growth of this culture of yeast in solid wort-gelatin (10 g. gelatin per 100 c.c. wort). The sterile solid medium was melted and seeded with about 100,000 yeast cells per c.c. 75 c.c. were poured into a bottle which was filled with pieces of glass tubing, the whole being previously sterilised. There still remained about 80 c.c. air space. The melted gelatin was cooled by rotating the bottle under a jet of cold water so that the gelatin as it solidified became distributed over the tubing.

The bottle was then connected to the manometer and the apparatus

exhausted. Measurements of the rate of fermentation were then taken and
the constant of growth calculated as in Table II

TABLE VI.

Temp. $= 15°$	t	$S + s$	$0.434\,K$
	-20	(0.1)	—
	0	$2.05\ (S)$	—
	4	3.8	0.067
	5	4.4	0.066
	6	5.15	0.067
	9.1	8.25	0.066
	9.4	8.65	0.067
	10.6	10.2	0.065
	10.8	10.4	0.065

Average $\overline{0.066}$ $T = 4.6$ hrs.

The rate of growth of a yeast colony developing in wort-gelatin from a
single cell therefore follows the logarithmic law. The experiments show that
retarding influences do not come into play up to the time the colony consists
of 200 yeast cells and probably the colony would grow regularly to a much
larger size. It is of interest to note that diffusion would play no controlling
part in determining the rate of fermentation until the colony consists of
several million yeast cells. Slator and Sand [1910] have made calculations
of the size of a yeast cell which would just ferment entirely the whole of the
sugar diffusing to it in a stationary liquid. At 30 the radius is calculated
to be 8×10^{-2} cm. The volume of such a yeast cell is 8 million times as
great as one of radius 4×10^{-4} cm. Diffusion under these conditions would
not be a limiting factor in the fermentation by a yeast colony until the
colony consists of 8 million cells.

The rate of growth of this culture of yeast in wort gelatin is appreciably
higher than in wort itself, the rates being 0·066 and 0·050 respectively.

The investigation shows the possibility of measuring rates of growth when
the organism is growing on a solid medium.

REFERENCES.

Slator (1906), *J. Chem. Soc.* **89**, 128.
—— (1908), *J. Soc. Chem. Ind.* **27**, 653.
—— and Sand (1910), *J. Chem. Soc.* **97**, 927.

XIX. THE IDENTITY OF TRIMETHYLHISTIDINE (HISTIDINE-BETAINE) FROM VARIOUS SOURCES.

By GEORGE BARGER AND ARTHUR JAMES EWINS.

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In a recent paper we showed [Barger and Ewins, 1911] that ergothioneine, a crystalline base containing sulphur, which was isolated from ergot by Tanret [1909], almost certainly possessed the constitution denoted by the formula 1:
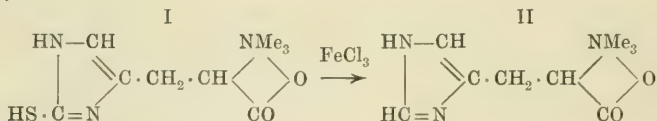
Ergothioneine was thus trimethylhistidine (histidine-betaine) containing a sulphur atom attached to a carbon atom of the glyoxaline ring. Further we showed that on oxidation with ferric chloride the sulphur atom was removed as in similar thiolglyoxaline derivatives [Pyman, 1911] and a new base trimethylhistidine or histidine-betaine (II) was produced. We described certain of its salts and pointed out the possibility of its natural occurrence. Kutscher [1910] had previously obtained a crystalline aurichloride of a base from commercial extract of mushrooms, and had stated that this base was possibly trimethylhistidine, since it possessed the formula $C_9H_{15}O_2N_3$ and gave a strong reaction with sodium p-diazobenzene sulphonate. As, however, no details were published regarding this salt, we were unable to determine whether our base was identical with Kutscher's.

A short time after our paper there appeared a publication by Reuter [1912] in which he described the isolation of histidine-betaine from *Boletus edulis,* and gave a description of certain of its salts. Among others he characterised two picrates: one, a monopicrate which was analysed, but to which no melting point was assigned, and another, melting at 206° which was not analysed, but which, from a picric acid determination, appeared to

be a dipicrate. The picrate of histidine-betaine obtained by us from ergo-thioniene melted at 123° and analysed well for the dipicrate. This difference was pointed out by Reuter and for some time we were at a loss for a satisfactory explanation. We now find that the apparent discrepancy was due to the fact that our melting point was determined on the air-dried salt, which contained two molecules of water of crystallisation: our analysis on the other hand was made with an anhydrous specimen of which the melting point was not at the time determined. Quite recently Kutscher [1912] succeeded in synthesising the betaine in question from α-chloro-glyoxaline-propionic acid and trimethylamine at 80°, and by means of the aurichloride established the identity of the synthetic base with the supposed trimethylhistidine previously isolated by him from mushrooms. On repeating Kutscher's synthesis we were able to isolate from the reaction product a small quantity of the picrate of the betaine which melted at 123° as we had found in the case of the base derived from ergothioneine. The two picrates were, indeed, in all respects identical.

At this juncture we communicated with Dr C. Reuter who very kindly supplied us with specimens of the two picrates prepared by him, together with a full description of these salts, for which we offer him our best thanks. We then found that the (anhydrous) dipicrate melting at 205°–206° described by Reuter, crystallised from water with two molecules of water of crystal-lisation, and, when air dry, melted at 123°–124°, and was then in all respects identical with the picrate obtained by us. The water of crystallisation could be removed by drying *in vacuo* over sulphuric acid, but only with some difficulty. The picrate thus obtained always showed signs of sintering at about 125° (doubtless owing to traces of water still adhering) but did not melt until 205°–206° as described by Reuter. The crystalline anhydrous salt could be readily obtained by recrystallising the picrate (dried *in vacuo*) from absolute alcohol, and then melted at 213°–214°. In a private com-munication Dr Reuter informed us that a purified specimen of his dipicrate melted at 212°–213°. We carried out determinations of the water of crystallisation present in our picrate (A) and that obtained by recrystallising Reuter's anhydrous dipicrate from water (B) with the following results.

A. 0·2061 (air dry) lost (*in vacuo* over H_2SO_4) 0·0106 $H_2O = 5·14$.
B. 0·1603   ,,   ,,   ,,   ,,   0·0083 $H_2O = 5·17$.
$C_9H_{15}O_2N_3 \cdot (C_6H_3O_7N_3)_2 \cdot 2H_2O$ requires $H_2O = 5·21$ per cent.

The complete identity of the two bases was established by the melting points of the two forms of the dipicrate and of the monopicrate (m.p. 201°–202°) which we prepared from our dipicrate according to Reuter's direction

(treating the aqueous solution of the dipicrate with one molecular proportion of sodium hydrate). The melting points of mixtures in all cases showed no depression.

There can be no doubt, therefore, that the trimethylhistidine (histidine-betaine) obtained by us from ergothioneine is identical with that obtained by Reuter from *Boletus edulis* and with the synthetic base obtained by Kutscher. This result affords a further confirmation of the constitution assigned by us, on other grounds, to ergothioneine.

For the isolation of the betaine of histidine we find that the preparation of the dipicrate is the most convenient method; this salt dissolves in about 25 parts of boiling water and readily crystallises in thin elongated rectangular plates with two molecules of water of crystallisation, and when air dry, melts at 123°–124°.

In our previous paper [1911] the melting point of the aurichloride of the betaine was given as 171°. As the quantity of material at our disposal was at that time very small (a few centigrams only) we were unable to analyse this salt, but now, with a further supply of material, we find that the gold salt when pure melts at 184°, in agreement with the melting point as given by Kutscher and by Reuter.

We also determined the rotation of the base recovered from Reuter's dipicrate with the following result.

Concentration of base (in aqueous solution as hydrochloride) = 0·39 per cent.

Actual rotation measured $\alpha_D = + 0\cdot40°$ (mean of 6 readings) in 2·2 d.m. tube. Whence $[\alpha]_D = + 46\cdot5°$.

In conclusion we may point out that the trimethylhistidines obtained from various sources are thus shown to be identical. This substance must be classed with the other naturally occurring betaines from amino-acids, such as those from glycine, proline, oxy-proline, and tryptophane (i.e. ordinary betaine, stachydrine, betonicine and hypaphorine respectively).

REFERENCES.

Barger and Ewins (1911), *J. Chem. Soc.* **99**, 2396.
Kutscher (1910), *Zentr. Physiol.* **24**, 775.
—— (1912), *Zentr. Physiol.* **26**, 869.
Pyman (1911), *J. Chem. Soc.* **99**, 2172.
Reuter (1912), *Zeitsch. physiol. Chem.* **78**, 167.
Tanret (1909), *J. Pharm. Chim.* [vi], **30**, 145.

XX. A NOTE ON THE METABOLISM OF NITROGENOUS SUGAR DERIVATIVES.

By JAMES ARTHUR HEWITT.

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It is very generally accepted that carbohydrates are of fundamental importance in the metabolism of nitrogenous foodstuffs. Loewi [1902], using protein decomposition products which no longer gave the biuret reaction, showed carbohydrate to be necessary for the maintenance of a nitrogen equilibrium. Lesser [1904] subsequently demonstrated the inability of fats to replace the carbohydrate. It has been observed further that glycosuria following excision of the pancreas in dogs is attended by increased excretion of nitrogen [Falta, Grote and Staehelin, 1910], and Taylor and Cathcart [1910] found phloridzin diabetes to be followed by disturbances of nitrogen metabolism. Finally, Falta and Gigon [1908] have concluded that carbohydrate is indispensable for the utilisation of protein, and it has been assumed that some compound is formed between the carbohydrate and the nitrogenous material [Lüthje, 1906].

In an attempt to determine the nature of this synthesis Spiro [1907] showed that the injection of glycine and fructose together gave rise to the appearance in the urine of a dicarboxylic acid normally absent. On the other hand much *in vitro* work has been done and various definite compounds of sugars and nitrogenous substances have been obtained. Lobry de Bruyn [1904] prepared compounds of sugar and ammonia, Schoorl [1900] a urea derivative of glucose, and Morrell and Bellars [1907] have synthesised a product from glucose and guanidine. It may be noted however that in some cases at least the reaction does not seem to be one of simple condensation [Irvine, 1909]. Little seems to have been done on the metabolism of substances of this nature.

Fabian [1899] found that glucosamine is with difficulty oxidised in the body and is in great part excreted unchanged. The extreme stability of this

compound and its great resistance to *in vivo* oxidation are somewhat unexpected; but in light of recent evidence brought forward by Irvine and Hynd [1912] for considering glucosamine to be not a simple derivative of glucose, but a ring compound containing a betaine grouping, Fabian's results are largely explained.

For the purposes of studying the metabolism of compounds such as those mentioned above, those referred to offer certain disadvantages; Lobry de Bruyn's products are somewhat indefinite, the urea derivative difficult to obtain in quantity, and most other compounds of this nature are highly unstable.

For the following experiments, glucose *p*-phenetidide, the condensation product of glucose and *p*-phenetidine, was selected on account of the ease of its preparation and its comparative stability to hydrolytic agents. At the boiling point, however, in solution it is readily hydrolysed by 0·2 % hydrochloric acid.

The preparation of glucose *p*-phenetidide was carried out according to the method devised by Irvine and Gilmour [1909].

The toxicity of the compound is also disputed [St Mostowski, 1909] (quoted from the above-mentioned authors).

White rats were used, the faeces and urine being collected separately in cages similar to those described by Schäfer [1912]. To prevent loss of nitrogen from the urine in the form of ammonia, known volumes of concentrated sulphuric acid were generally added to the receiving vessel previous to collection. The glucose *p*-phenetidide was administered either in the food (bread and milk *ad libitum*), or less often by intra-peritoneal injection. Quantities of from 0·1 to 1 g. (usually 0·5 g.) were given daily. The intake and output of nitrogen were determined both before and during the experiment, but no alteration in the nitrogen equilibrium was observed. The presence of glucose *p*-phenetidide in the food caused no variation in the amount consumed *per diem* which could not be accounted for by the normal difference from day to day.

The urine of those animals receiving the compound was without exception of a much darker colour than that of the controls or of themselves before or after the feeding. Discontinuance of feeding at once restored the normal light yellow colour. The colour is not caused by hydrolysis taking place in the urine by means of the acid present, for when the urine was collected alone the colouration persisted[1].

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[1] Oxidation of glucose *p*-phenetidide by means of hydrogen peroxide in neutral solution produces a brownish black syrup of unknown composition.

Injection of 0·1 g. produced similar results, and moreover in each case a substance displaying active reducing properties was excreted in the urine. Greater reduction of Fehling's solution, Barfoed's solution and Nylander's reagent occurred after previous boiling of the urine with mineral acid. In one case following injection, a compound was obtained from the urine by means of phenylhydrazine, which in microscopic appearance resembled the phenylosazone of glucose, but owing to the small quantity confirmation could not be obtained.

It was mentioned above that the toxicity of glucose p-phenetidide was disputed, St Mostowski denying the general conclusion that it is poisonous (we are unaware however of the quantities administered by this author).

In these experiments it is shown that in quantities up to 1 g. per diem it produces no ill effects in rats of from 220 to 250 g. body weight. Experiments with a view to determining the effects of larger doses were not performed owing to scarcity of material.

Injection of p-phenetidine however is followed by marked results. The p-phenetidine cannot be given by feeding owing to the animals refusing to eat all food containing it.

In a cat 4 c.c. of an 8 % solution caused a large and rapid fall of blood pressure which returned to normal within a minute and a half and remained steady and regular until death ensued ten minutes later. Post-mortem examination revealed extensive intra-vascular clotting.

It seems probable that much of the glucose p-phenetidide escapes hydrolysis or is rendered innocuous by conversion into some other substance, from the facts that in doses of 4 c.c. of an 8 % solution or 0·32 g. per 2·5 kilo body weight, p-phenetidine is highly toxic, and that glucose p-phenetidide is harmless when given in amounts of 1 g. per 220 g. or 4·5 g. per kilo body weight, equivalent, if complete hydrolysis took place, to about 2 g. p-phenetidine.

Summary.

1. After administration of glucose p-phenetidide by mouth or by injection, a reducing substance is excreted by the urine.

2. No effects on nitrogen metabolism are produced.

3. In amounts up to 1 g. per 220 to 250 g. body weight per diem glucose p-phenetidide is not toxic.

4. Some of the glucose p-phenetidide escapes oxidation in the body or is converted into a substance of non-poisonous nature.

5.　In amounts of 0·32 g. per 2·5 kilo body weight (or 0·108 g. per kilo) *p*-phenetidine is highly toxic.

The author would like to express his indebtedness to Dr Cathcart of Glasgow University for suggesting the line of research, and also to Professor Irvine and to Professor Herring of St Andrews University for kindly advice and criticism.

REFERENCES.

Fabian (1899), *Zeitsch. physiol. Chem.* **27**, 167.
Falta, Grote and Staehelin (1907), *Beitr. chem. Physiol. Path.* **10**, 197.
—— and Gigon (1908), *Biochem. Zeitsch.* **13**, 267.
Irvine (1909), *Biochem. Zeitsch.* **22**, 357.
—— and Gilmour (1909), *J. Chem. Soc.* **95**, 1150.
—— and Hynd (1912), *J. Chem. Soc.* **101**, 1135.
Lesser (1904), *Zeitsch. Biol.* **45**, 497.
Lobry de Bruyn (1904), *Maandbl. Natuurw.* **18**, 85.
Loewi (1902), *Arch. exp. Path. Pharm.* **48**, 803.
Lüthje (1906), *Arch. ges. Physiol.* **113**, 547.
Morrell and Bellars (1907), *J. Chem. Soc.* **91**, 1010.
Mostowski, St (1909), *Bull. Akad. Sci. Cracow*, 641.
Schäfer (1912), *Quart. J. Exp. Physiol.* **5**, 204.
Schoorl (1900), *Akad. Wetensch. Amsterdam*, 410.
Spiro (1907), *Beitr. chem. Physiol. Path.* **10**, 247.
Taylor and Cathcart (1910), *J. Physiol.* **41**, 276.

XXI. AN ATTEMPT TO ESTIMATE THE VITAMINE-FRACTION IN MILK.

By CASIMIR FUNK, *Beit Memorial Research Fellow.*

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The isolation of the beri-beri vitamine from milk [Funk, 1912, 1], together with the important fact observed by Andrews [1912], that infantile beri-beri occurs when the children are fed by mothers suffering from beri-beri, suggest a new line of investigation into the etiology of infantile scurvy and rickets. The problems which present themselves in connection with these investigations have been already set forth [Funk, 1912, 2] and will be fully treated in an account in the *Ergebnisse der Physiologie*, Vol. 13. These problems are briefly the following. (1) What is the normal amount of vitamines in milk of different species, including the human? (2) Is there a definite relationship between the amount of vitamines secreted in the milk and that ingested in the food? This point is of special interest because of the periodical appearance of rickets in winter and disappearance in summer, a period when the cows return to pasture. (3) What effect have boiling and pasteurisation on the vitamine content of milk? All these points await their solution until a method for determining such small quantities is available. The present inquiry shows that the ordinary chemical methods for estimating vitamines can hardly suffice and attention at present must therefore be directed to colorimetric methods.

Nevertheless the results described below give an idea of the amount of vitamines possibly present in milk. The whole vitamine fraction (nitrogen precipitated by phosphotungstic acid in the alcoholic extract of the dried milk) from 1 litre of milk amounts to 1–2·5 mgr. nitrogen. This would correspond to 1–3 cg. vitamine ($C_{17}H_{20}O_7N_2$). After the elimination of the vitamine-fraction the residual nitrogen amounts to 20–50 mgr. per litre milk. This residual nitrogen represents in all probability allantoin; assuming this to be correct, one litre of milk contains 0·06–0·15 grm. allantoin, a figure in good agreement with that obtained by Ackroyd [1911] by means of a direct method, namely 0·199 grm.

The results further indicate that for a chemical investigation of the vitamines of milk it will be necessary to start on a large amount of milk. At the same time they show that the artificial protein-free milk, such as was used by Osborne and Mendel [1912] in their experiments on growth, differs from true protein-free milk in lacking these nitrogenous substances. It is therefore quite conceivable, as the experiments of Hopkins and Neville [1913] point out, that these substances play an important part in the process of growth.

As the figures below show, the milk after removal of fat by centrifuging has lost about 50% of vitamine and allantoin.

The milks used in these experiments were from London dairies. For each experiment a litre was evaporated in vacuo at 30°, using a distilling flask constructed of two parts from which therefore the residue could be quantitatively removed. The process of drying the residue was hastened in the last stages by an addition of alcohol. The residue was powdered in a mortar and dried in a vacuum desiccator to constant weight. The fine powder was then shaken with a definite amount of alcohol (400 c.c.) in a shaking machine for two hours. The extract was filtered and of the filtrate an aliquot part was evaporated in vacuo. The residue was extracted with water and the extract transferred to a measuring flask. The watery extract was filtered and the filtrate found in every case to be entirely free from proteins. A measured quantity was acidified slightly with sulphuric acid and precipitated with 10% phosphotungstic acid. After standing for 48 hours the precipitate was filtered and washed with dilute sulphuric acid. In accordance with my previous results this phosphotungstic acid precipitate can be regarded as the vitamine-fraction. Both in the precipitate and filtrate the nitrogen was estimated by Kjeldahl, N/20 normal solutions being used for titration.

		Weight of residue	Nitrogen of the vitamine fraction in mgr. (phosphotungstic acid precipitate)	Residual nitrogen in mgr. (phosphotungstic acid filtrate)
1.	Centrifuged milk	100	2·54	50·9
	Control	99·5	2·46	49·6
2.	Uncentrif.	133	1·7	30·7
	Centrif.	98	1·53	16·8
3.	Uncentrif.	127	2·5	22·7
	Centrif.	90	1·4	12·1
4.	Uncentrif.	129	1·96	29·6
	Centrif.	92	1·2	15·3
5.	Uncentrif.	132	2·4	49·2
	Centrif.	96	1·6	27·3
6.	Centrif.	89	1·06	22·4
7.	Centrif.	105	1·83	44·9

In this connection the behaviour of allantoin when boiled with Ruhemann's reagent (triketohydrindene hydrate) should be noted. According to Abderhalden and Schmidt [1911] allantoin when boiled for one minute with Ruhemann's reagent does not give any colouration. But although this is so, if the boiling be continued for 2–3 minutes a violet colouration appears and this reaction might be used as a test for allantoin. Since the crude product obtained by oxidation of uric acid by permanganate gave the reaction at once, probably due to admixture with allanturic acid, and allantoin is known to be destroyed by boiling, it was thought that a decomposition product of allantoin, probably allanturic acid, was the cause of this reaction. A sample of pure allantoin was therefore boiled with PbO_2 and another with water. Both samples gave a strong allanturic acid reaction after a certain time of boiling, the sample with PbO_2 giving however the stronger reaction. The reaction seemed to disappear after prolonged boiling. The reaction may also be obtained from hydantoin.

It is interesting to note, that we have in allantoin an example of a substance, which like vitamine is destroyed by boiling.

REFERENCES.

Abderhalden and Schmidt (1911), *Zeitsch. physiol. Chem.* **72**, 87.
Ackroyd (1911), *Biochem. J.* **5**, 400.
Andrews (1912), *Philipp. J. Sc.* **7**, 67.
Funk, Casimir (1912, 1), *J. Physiol.* **45**, 75.
—— (1912, 2), *J. State Med.* **20**, 341.
Hopkins and Neville (1913), *Biochem. J.* **7**, 97.
Osborne and Mendel (1912), *Zeitsch. physiol. Chem.* **80**, 307; *J. biol. Chem.* **13**, 243.

XXII. THE ENZYMES OF WASHED ZYMIN AND DRIED YEAST (LEBEDEW). I. CARBOXYLASE.

By ARTHUR HARDEN.

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When zymin is thoroughly washed with water a residue is left, which no longer has the power of fermenting glucose, but regains this power when the washings are added to it, even when the latter have been boiled [Harden and Young, 1910]. The dried yeast used by Lebedew behaves in a precisely similar manner [Euler and Bäckström, 1912].

It is therefore a matter of considerable interest to ascertain how far this treatment affects the various enzymes which are known to exist in zymin and dried yeast. It can thus be ascertained whether these enzymes are themselves soluble and whether if insoluble they require for their action the presence of a soluble substance of the nature of a coenzyme.

It is also probable that some light may be thrown on the possible function of some of these enzymes in the process of alcoholic fermentation.

Carboxylase.

Since the discovery by Neuberg and Hildesheimer [1911] of the unexpected fact that yeast, yeast juice and zymin readily and rapidly decompose pyruvic acid and other α-ketonic acids with evolution of carbon dioxide and formation of an aldehyde, the opinion has been expressed in many quarters that pyruvic acid may form a stage in the enzymatic decomposition of glucose into alcohol and carbon dioxide [Neubauer and Fromherz, 1911 ; Neuberg and Kerb, 1912 ; Kostytschew, 1912 ; Lebedew 1912].

On the other hand it is possible that carboxylase is quite independent of the enzymes of alcoholic fermentation, its function being that of decomposing the α-ketonic acids formed by the deaminisation of the α-amino-acids (see Neubauer and Fromherz, 1911).

In order to examine the action of washed yeast preparations on pyruvic acid, experiments were made not only on the free acid but also on the

sodium salt in presence of weak inorganic acids, by the aid of which the acidity was diminished, whilst at the same time the whole or almost the whole of the carbon dioxide produced was evolved.

The evolution of carbon dioxide was observed by the aid of the apparatus previously described [Harden, Thompson and Young, 1910]. No quantitative estimations of acetaldehyde were made, but its presence was proved by the reaction with Schiff's reagent in all cases in which an evolution of carbon dioxide was observed. Control experiments were at the same time carried out with glucose and phosphate. It was thus found that the residue obtained by washing zymin and dried yeast (Lebedew) until they could no longer ferment glucose, was capable of decomposing pyruvic acid quite readily. The interesting fact is thus ascertained that carboxylase does not require the presence of a coenzyme removable by washing in order to exert its characteristic reaction on pyruvic acid. This result does not however allow any definite conclusion to be drawn as to the possible function of carboxylase in alcoholic fermentation. It can only be concluded that if the decomposition of pyruvic acid actually be a stage in the alcoholic fermentation of glucose, the soluble coenzyme is required for some change precedent to this, so that in its absence the production of pyruvic acid cannot be effected. The following experiments illustrate the results obtained. The solutions throughout were saturated with carbon dioxide at the temperature of the bath before the commencement of incubation.

Exp. 1. A sample of Schroder's dried yeast (nach Lebedew) was washed 3 times on the centrifuge with water and then brought into acetone and dried. The following experiments were then made.

(a) 2 g. washed and acetoned yeast + 10 c.c. 1 per cent. pyruvic acid + 40 c.c. H_2O.

(b) 2 g. washed and acetoned yeast + 25 c.c. 1 per cent. pyruvic acid + 25 c.c. H_2O.

(c) 2 g. washed and acetoned yeast + 25 c.c. 1 per cent. pyruvic acid + 5 c.c. 0·3 molar Na_2HPO_4 + 20 c.c. H_2O.

(d) 2 g. washed and acetoned yeast + 0 pyruvic acid + 5 c.c. 0·3 molar Na_2HPO_4 + 45 c.c. H_2O + 2 g. glucose.

These were incubated at 25° and the evolution of CO_2 measured.

Time	(a)	(b)	(c)	(d)
1 hr. 50 mins.	3·5	1·6	18·6	1·9

The action of the washed yeast on glucose was extremely small (d) and very little fermentation of the free pyruvic acid (a and b) occurred. In presence of sodium phosphate however a considerable evolution of CO_2

occurred (18·6 c.c.) and a strong reaction for acetaldehyde was given by the filtrate. The small effect on free pyruvic acid is ascribed to the effect of the acidity of the solution on the enzyme ; in presence of sodium phosphate, on the other hand, the acidity is mainly due to NaH_2PO_4 formed by interaction of the pyruvic acid with the Na_2HPO_4.

Exp. 2. A similar experiment was made with similar results using the same washed and acetoned yeast, but washing it again 3 times to remove the last trace of coenzyme.

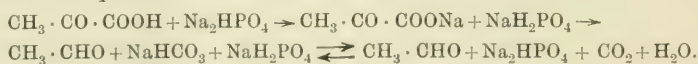10 g. of acetoned yeast were washed and made up to 85 c.c.

(a) 25 c.c. yeast suspension + 21 c.c. 1 per cent. pyruvic acid + 7 c.c. H_2O.

(b) 25 c.c. yeast suspension + 21 c.c. 1 per cent. pyruvic acid + 7 c.c. 0·3 molar Na_2HPO_4 (approx. equivalent to the pyruvic acid).

(c) 25 c.c. yeast suspension + 21 c.c. H_2O + 7 c.c. 0·3 molar Na_2HPO_4 + 2 g. glucose.

Time	(a)	(b)	(c)
55 mins.	4·1	22·0	1·2 c.c.

Here again the action on the free acid is small, whereas a considerable evolution of CO_2 occurs in presence of phosphate. The amount produced is really greater than that evolved since when pyruvic acid and sodium phosphate are present in equivalent amount a portion of the CO_2 is retained as $NaHCO_3$ in equilibrium with NaH_2PO_4.

$$CH_3 \cdot CO \cdot COOH + Na_2HPO_4 \rightarrow CH_3 \cdot CO \cdot COONa + NaH_2PO_4 \rightarrow$$
$$CH_3 \cdot CHO + NaHCO_3 + NaH_2PO_4 \rightleftarrows CH_3 \cdot CHO + Na_2HPO_4 + CO_2 + H_2O.$$

Accordingly, the combined CO_2 was determined after incubation and, in a separate sample, before incubation and it was thus found that 12·9 c.c. of CO_2 were retained and should be added to the amount observed in (b), making a total production of 34·9 c.c.

Exp. 3. The effects of neutral potassium citrate and of excess of free boric acid in presence of the sodium salt of pyruvic acid were tried. The yeast employed was a similar preparation to that used in Exp. 2.

(a) 25 c.c. yeast suspension + 21 c.c. 1 per cent. pyruvic acid + 7 c.c. 0·3 M. potassium citrate neutral to phenolphthalein.

(b) 25 c.c. yeast suspension + 21 c.c. H_2O + 7 c.c. 0·3 M. sodium phosphate + 2 g. glucose.

(c) 25 c.c. yeast suspension + 21 c.c. H_2O + 7 c.c. 0·3 M.K. citrate.

(d) „   „   + 21 c.c. 1 per cent. pyruvic acid + 2·4 c.c. N. KHO + 3 g. H_3BO_3.

Time	(a)	(b)	(c)	(d)
1 hr. 25 mins.	4·5	1·3	1·0	25·6

The sample of yeast had practically no action on glucose (*b*), comparatively little on pyruvic acid in presence of citrate (*a*) and a large action in presence of free boric acid (*d*). This result therefore is in agreement with the idea that the action of the enzyme is greatly inhibited by acid, citric acid being much stronger than boric acid.

Exp. 4. 10 g. dried yeast was washed 3 times and made to 100 c.c.

(*a*) 25 c.c. yeast suspension + 25 c.c. 1 per cent. pyruvic acid + 2·5 c.c. N. KHO + 3 g. boric acid + toluene.

(*b*) 25 c.c. yeast suspension + 21 c.c. H_2O + 5 c.c. 0·3 M. Na_2HPO_4 + 2 g. glucose + toluene.

Time	(*a*)	(*b*)
1 hour	21·04	0·2 c.c.

Here again a good fermentation of pyruvic acid is produced by a sample of washed yeast incapable of fermenting glucose, the total evolution from which in 18 hours was only 0·4 c.c.

Exp. 5. 20 g. zymin (Schroder) were washed 3 times and made to 100 c.c.

(*a*) 25 c.c. yeast suspension + 22·5 c.c. H_2O + 2 g. glucose + 5 c.c. 0·3 M. Na_2HPO_4.

(*b*) „ „ + 25 c.c. 1 per cent. pyruvic acid + 2·5 c.c. N. KHO + 3 g. boric acid.

| Time	(*a*)	(*b*)
43 mins.	17·8	0·8
18 hours	22·7	0·8

Washed zymin therefore has the same action as washed dried yeast.

SUMMARY.

Zymin and dried yeast (Lebedew) after being freed from coenzyme by washing and thus rendered incapable of fermenting glucose, readily decompose pyruvic acid into carbon dioxide and acetaldehyde, provided that the acidity of the solution is kept low.

REFERENCES.

Euler and Bäckström (1912), *Zeitsch. physiol. Chem.* **77**, 394.
Harden, Thompson and Young (1910), *Biochem J.* **5**, 230.
—— and Young (1910), *Zentr. Bakt. Par.* II. **26**, 178.
Kostytschew (1912), *Zeitsch. physiol. Chem.* **79**, 359.
Lebedew (1912), *Ber.* **45**, 3256.
Neubauer and Fromherz (1911), *Zeitsch. physiol. Chem.* **70**, 326.
Neuberg and Hildesheimer (1911), *Biochem. Zeitsch.* **31**, 170.
—— and Kerb (1912), *Zeitsch. Gärungsphysiol.* **1**, 114.
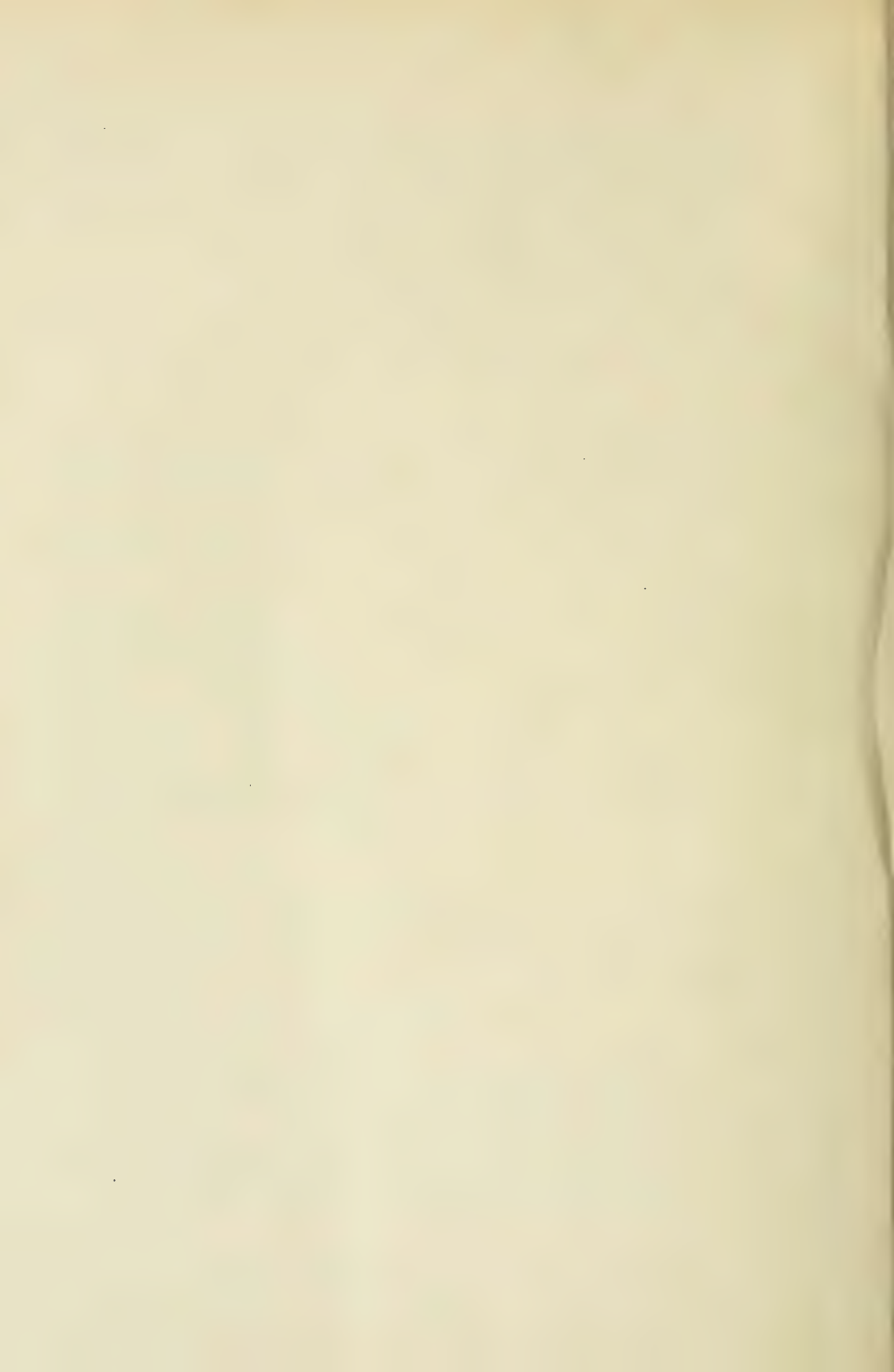
XXIII. THE CRITICAL SOLUTION POINT OF URINE.

By WILLIAM RINGROSE GELSTON ATKINS AND
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From Trinity College, Dublin.

In order to determine the efficiency of a kidney as an excretory organ, the most exact method is the determination of the total solid content of the urine (neglecting colloids), as shown by the depression of its freezing-point below that of pure water. The examination of urine and blood by this method has proved very useful and reliable, but the time required for carrying it out constitutes a serious drawback when only one or two specimens are to be examined.

The following method was brought forward by one of us [Atkins, 1908] in the hope that it might be of service in cases where great accuracy is not essential and saving of time an advantage. Since no special apparatus is required and ice is not employed the usefulness of critical solution point determinations as a method of clinical examination is much increased especially for work in warm climates.

The present paper gives an outline of the method of such determinations, as the subject is a comparatively recent one. The data of the former paper are extended and the influence of the various constituents of the urine upon the critical solution point is traced out.

OUTLINE OF CRITICAL SOLUTION POINT THEORY.

(*a*) *Two pure substances.* Consider two liquids not completely miscible at room temperatures, such as phenol and water. If small quantities of these two substances are shaken together a white emulsion is obtained; this on standing separates into two layers, a lower one, consisting of phenol with some water dissolved in it, and an upper one of water with some dissolved phenol. If the temperature be now raised, the quantity of each dissolved in

the other increases till a temperature is reached at which the two become completely miscible; on cooling even a tenth of a degree separation again occurs, a white emulsion is formed, and on standing the two layers separate out as before. The temperature, at which the two layers become homogeneous, depends upon the proportions of water and phenol initially present, and rises in the case of water as the amount of phenol is increased until a mixture is reached which remains in the two phases at a higher temperature than any other mixture of the two pure components.

The composition of this mixture is termed the "critical concentration," and the temperature at which the liquid becomes homogeneous is called the "critical solution temperature." This temperature is accordingly that at which the two phases have the same composition and the same properties. At the critical concentration and critical solution temperature the volumes of the two phases, water in phenol and phenol in water, are equal.

At the critical temperature the two phases have the same surface tension, and are therefore completely miscible; at a very slightly lower temperature the surface tensions of the two phases are almost equal, consequently the meniscus between them is flat. As the homogeneous mixture cools, at a temperature very little above the critical temperature a beautiful opalescence appears, which is blue by reflected light and brown by transmitted.

If now a series of mixtures of water and phenol be taken and the temperatures at which they become homogeneous be determined, and the values thus obtained be plotted as ordinates, taking the corresponding percentage compositions of the mixtures as abscissae, a curve is obtained, the highest point of which is the critical solution point [Fig. 1, Schreinemakers, 1899]. This curve is somewhat flat at the top, hence the composition of the mixture may be slightly altered to one side or the other of the critical concentration without altering the temperature of miscibility more than about 0·1° C. The blue opalescence is still obtained, but it is not as beautiful as at the critical solution point itself.

(b) *Effect of a third substance.* The addition of a very small quantity of a third substance to any mixture of water and phenol raises the temperature at which the two phases become homogeneous, provided the added substance be freely soluble in water and almost or quite insoluble in phenol, or *vice versa.* If the added substance be freely soluble in both, then the temperature at which a homogeneous mixture is obtained is lowered. Thus by adding a small quantity of sodium oleate to a mixture of phenol and water, it is possible to obtain a very strong solution of phenol, homogeneous at room temperatures. Lysol, the disinfectant, is prepared in a similar way.
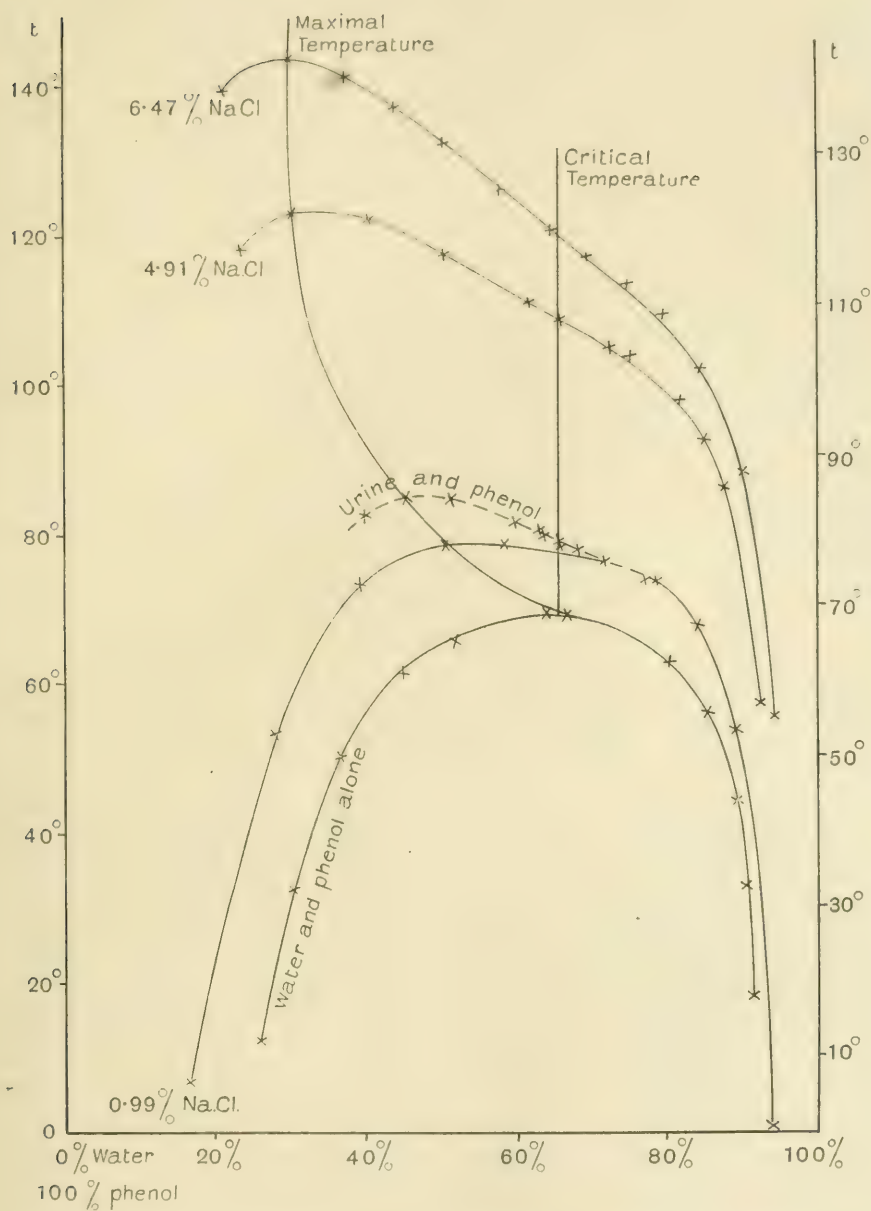
Fig. 1. In part after Schreinemakers. The dotted line shows the data for urine given in Table I.

In the case of a substance much more soluble in water than in phenol, if curves (Fig. 1) constructed as described be plotted when one of the pure liquids is replaced by a dilute solution of the third substance in that liquid, the general outline of the original curve is followed, the ordinates of temperature being everywhere greater and markedly so in what is termed the crest region. In each of these curves there is a critical point, but it no longer coincides with the maximum temperature as is the case when two pure components are concerned; accordingly it must be recognised by the beautiful opalescence and equality of volume of the layers.

The rise of critical temperature for dilute solutions, say one per cent., is roughly proportional to the number of gram-molecules of the substance added, with regard to its own solvent. This rise is very considerable, for an amount of solid sufficient to depress the freezing point of water $0.5°$ may raise the critical temperature of a mixture of water and phenol by as much as 4—5°. This rise therefore which can readily be determined to $0.1°$ gives about the same accuracy as a freezing point measured to $0.01°$ with greater facility and simple apparatus.

Application of the Method to Urine.

If in the preceding experiments urine, which is an aqueous solution of certain compounds, be substituted for water, a considerable rise in the critical solution temperature is found. This varies with the concentration of the urine.

A specimen of normal urine was examined throughout the whole curve, which was found to be similar to that given by a single substance when added to a water and phenol mixture, as shown by the broken line in Fig. 1. The method of procedure was as follows :—Weighed quantities of phenol and urine were mixed in test tubes, and the temperatures at which they became homogeneous were noted. A thermometer graduated in tenths from 50—100° is suitable, but for clinical work one graduated in degrees is sufficiently accurate. It is not so rough a measurement as it may appear to those accustomed to Beckmann thermometers, for the temperature differences to be measured are nearly ten times as great as those met with in freezing point work. The data from which the broken line in Fig. 1 was constructed are given below.

Inspection of the above table shows that the critical solution point of the phenol-urine mixture is at $79.7°$, the critical concentration being 36 per cent. phenol. The critical solution point is by no means at the crest of the curve,

but is recognised by the characteristic opalescence. The critical solution temperature of the phenol-water mixture similarly determined is 66·5°. Thus the rise produced by the solids dissolved in the urine amounts to 13·2°.

TABLE I.

Percentage weight, phenol	Percentage weight, urine	Temperature of homogeneity	Notes
22·42	77·58	73·4	——
31·31	68·69	78·0	——
33·99	66·01	78·3	Opalescence.
34·28	65·72	78·7	Good opalescence.
35·93	64·07	79·7	Best opalescence.
36·48	63·52	80·0	Good opalescence.
39·78	60·22	81·2	No opalescence.
48·45	51·55	84·3	——
54·22	45·78	84·7	Maximum temperature = crest
60·06	39·94	82·2	point.

Having established the similarity between the curve afforded by urine and by a single added substance it is quite sufficient to determine the critical solution point of any urine with phenol in the following manner. Dry crystalline phenol is placed in a test tube to a depth of about 1 cm., and

TABLE II.

Normal Urine.

No.	Δ	R_c	$\dfrac{R_c}{\Delta}$
1	0·98°	7·7°	7·86
2	1·07	9·4	8·78
3	1·07	9·7	9·06
4	1·16	8·5	7·34
5	1·41	10·4	7·39
6	1·45	11·6	8·00
7	1·47	11·3	7·68
8	1·47	12·5	8·50
9	1·63	11·9	7·28
10	1·67	12·3	7·36
11	1·71	14·0	8·19
12	1·73	12·5	7·21
13	1·79	15·0	8·38
14	1·85	16·3	8·80
15	1·87	11·8	6·31
16	1·87	15·9	8·71
17	1·87	15·5	8 28
18	1·87	16·0	8·55
19	1·92	15·3	7·96
20	2·48	17·4	7·00
		Mean	7·93

slightly more than enough urine to cover it is added from a pipette. The mixture is warmed and, if the critical opalescence does not appear on cooling, urine is added in small quantities till a good opalescence is shown. In this manner one proceeds from a too concentrated mixture of phenol to one of the critical composition without the trouble of weighing the components. These determinations can be very rapidly made, the time depending on how nearly the critical concentration has been reached at the first trial.

A number of normal urines were examined by this method, their freezing points being also obtained in the usual manner, and opposite the depression Δ the rise in critical solution temperature R_c has been tabulated. A third column gives the ratio R_c/Δ, as shown in Table II.

It is clear from the above figures that there is a roughly constant ratio between the two sets of numbers. The fluctuations however are considerably beyond the range of any likely experimental error, though with highly coloured urines it is not easy to decide as to the most characteristic opalescence. (In practice it is permissible, within limits, to dilute such specimens, but in obtaining the above results this was avoided.) Thus in Nos. 15–18, which have the same freezing point, it is seen that in three cases the critical solution points are within $0.5°$, whereas the fourth is widely divergent. As it seemed probable that such cases were to be explained by some appreciable differences in the relative proportions of the constituents of the urines in question, the most important of these were estimated in a number of specimens. Furthermore, in order to study the effect of one constituent alone, this was added in varying quantities to the analysed urine. Table III shows the analyses of Nos. 4, 5, 6, 10, 15 of Table II, expressed as grams per 100 cc.

TABLE III.

No.	Cl′	PO_4'''	SO_4''	NH_4^+	Urea	Uric acid	Total N	Δ	R_c	$\dfrac{R_c}{\Delta}$
4	0·351	0·225	0·173	0·059	1·612	0·015	0·909	1·16	8·5	7·34
5	0·270	0·289	0·238	0·056	1·902	0·021	1·074	1·41	10·4	7·39
6	0·721	0·227	0·138	0·049	1·542	—	0·843	1·45	11·6	8·00
10	0·756	0·222	0·236	0·078	1·854	—	1·080	1·67	12·3	7·36
15	0·596	0·270	0·251	0·097	2·252	—	1·397	1·87	11·8	6·31
					Dextrose					
10 a	0·453	0·133	0·141	0·047	1·113	2·02	0·648	1·21	10·0	8·26
10 b	0·226	0·066	0·070	0·023	0·556	3·53	0·324	0·86	6·8	7·86

The analyses were made in the usual manner as described by Neubauer-Huppert, *Analyse des Harns*. The first urea estimations were made by the method of Benedict and Gephart [1908] while in the later ones Folin's new method was employed [1912]. The latter gives slightly lower results than the former, owing probably to the fact that in it uric acid is not hydrolysed.

In considering what effect the various constituents will have upon the critical solution point, it must be remembered that whereas the salts are far more soluble in water than in phenol, urea is soluble in both. It has been shown, moreover, that urea forms a compound with phenol; this, however, is very largely dissociated [J. C. Philip, 1903]. Accordingly, though an increase of urea causes an increase in the depression of freezing point, it causes a decrease in the critical solution point temperature as shown later on.

The analyses recorded show that Nos. 4, 5, 10 are very similar in composition, the only notable difference being the larger chloride content of No. 10. It may be seen that the ratio R_c/Δ is for these specimens quite constant. In No. 6, while the chloride percentage is high, that of urea is relatively low and R_c/Δ has increased; whereas, in No. 15, the diminished amount of chloride and the large rise in urea and total nitrogen combine to lower R_c/Δ very considerably.

To study the effect of increase in chloride, the sodium salt was added in various proportions to No. 4, as shown in Table IV.

TABLE IV.

Chloride alone varied.

No.	Cl'	Δ	R_c	$\dfrac{R_c}{\Delta}$
4	0·351	1·158°	8·5°	7·34
4x	0·703	1·472	12·8	8·70
4y	1·055	1·797	18·0	10·02
4z	2·111	2·780	29·7	10·69

Here the effect of the chloride in raising the critical solution point is well brought out by the increased values of R_c/Δ. Separate studies of the other salts were not made, as, owing to their solubility relations, their effects must be much the same as that of the chloride. Also they seem to vary less and occur in smaller quantities.

The addition of pure urea was next studied by adding weighed quantities to No. 5. This, of course, alters the amount of total nitrogen also.

TABLE V.

Urea and total nitrogen varied.

No.	Urea	Total nitrogen	Δ	R_c	$\dfrac{R_c}{\Delta}$
5	1·902	1·074	1·408°	10·4°	7·39
5x	2·446	1·328	1·527	10·2	6·68
5y	2·992	1·583	1·721	9·5	5·52
5z	4·628	2·345	2·157	7·8	3·62

Thus while urea alone lowers the critical solution temperature, its effect in urine is to lessen the rise produced by the saline bodies. Consequently a specimen which contains more urea than is usual, will have a smaller value for R_c/Δ. Thus the fluctuations in this ratio, as seen in Table II, are explained by the mutually counteracting effects of the salts and urea upon the critical solution point.

PATHOLOGICAL URINES.

It remains to consider to what extent pathological conditions of the urine may alter the constancy of the ratio R_c/Δ. The simplest case is, perhaps, that in which the urine is normal in its constituents, but dilute. Table VI shows the results obtained by examining both normal urines after dilution and pathological urines.

TABLE VI.

Normal diluted and pathological dilute urines.

No.	Nature of specimen	Δ	R_c	$\dfrac{R_c}{\Delta}$
21.	Normal diluted	0·33°	3·2°	9·70
22.	,, ,, ...	0·44	4·7	10·70
23.	,, ,, ...	0·87	7·6	8·74
24.	Pathological	0·70	7·0	10·00
25.	From right kidney of A, by catheterisation ...	0·47	4·5	9·58
26.	From left kidney of A, ,, ,, ...	0·73	7·0	9·58
27.	From both kidneys of B, morning; nothing but water since previous evening	0·63	5·5	8·73
28.	From right kidney of B, by catheterisation at 1 p.m. Conditions as above. Albumin present ...	0·24	2·5	10·42
29.	From left kidney of B, by catheterisation. Conditions as above. No albumin	0·30	3·0	10·00
			Mean	9·72

These figures show that in very dilute urines the salts have a somewhat greater effect in raising the critical solution temperature than they have in those of average concentration. It is, however, to be noted that with such small values of R_c, an error of 0·1° affects the value of R_c/Δ very considerably. The similarity of R_c/Δ in Nos. 25 and 26, and also in Nos. 28 and 29, is noteworthy, and gives reason for supposing that the two kidneys were in each case excreting fluids almost identical in composition though not in concentration. The identity in composition of the urine from the right and left kidney is now completely established as a normal occurrence, though the earlier work seemed to point to the opposite conclusion [Casper and Richter].

A small number of other pathological urines were examined, as shown in Table VII.

TABLE VII.

No.	Nature of specimen	Δ	R_c	$\dfrac{R_c}{\Delta}$
30.	Chronic parenchymatous nephritis, albumin present ...	1·12°	9·3°	8·30
31.	Specimen with albumin, which was removed by boiling	1·04	9·3	8·94
32.	Alkaline, much albumin	1·27	11·0	8·66

In the above three instances, high values of R_c/Δ were obtained, probably due to a preponderance of salts in the urines. Albumin, being a colloid, can hardly be expected to have any perceptible effect either in raising or lowering the critical solution temperature. In the presence of the phenol in the critical concentration (36 %), the albumin in any specimens met with was not precipitated, even by boiling for five minutes.

DIABETIC URINES.

As the presence of dextrose has a great effect upon the freezing point it was thought well to see what alteration in the critical solution temperature its presence in urine would cause. As it is somewhat soluble in phenol, its addition to a phenol-water mixture was first studied. Results are shown in Table VIII.

TABLE VIII.

Dextrose added to phenol-water system.

Dextrose, grams per 100 cc. water	Δ	R_c	$\dfrac{R_c}{\Delta}$
5·05	0·56°	1·7	3·04
4·04	0·42	1·0	2·38
2·02	0·21	0·4	1·90
1·01	0·10	0·3	3·00
0·50	0·05	−0·1	−2·00

It will be seen that the addition of dextrose causes a relatively small rise in the critical solution temperature, the last figure being obviously due to experimental error. As before pointed out, with low values of R_c small errors cause the ratio R_c/Δ to fluctuate greatly. The effect of dextrose in a urine will, therefore, be to lower the value of R_c/Δ, for though it causes a rise in R_c yet this is not so great as is the case with the salts. To test this point further, No. 10 was diluted by the addition of dextrose solution, so as to have the

composition recorded in Nos. 10 a and 10 b, in Table III. Also dextrose was weighed out and made up to 100 cc. with No. 15, so that the resulting solutions had very closely the composition of No. 15, but not exactly on account of the volume occupied by the dissolved dextrose. The figures obtained are shown in Table III and in Table IX.

TABLE IX.

Dextrose added to phenol-urine system.

No.	Dextrose, grams per 100 cc. urine	Δ	R_c	$\dfrac{R_c}{\Delta}$
10	—	1·67°	12·3°	7·36
10 a	2·02 (No. 10 diluted)	1·21	10·0	8·26
10 b	3·53 (No. 10 diluted)	0·86	6·8	7·86
15	—	1·87	11·8	6·31
15 a	1·08	1·98	11·3	5·71
15 b	5·40	2·43	12·3	5·06

Here it is seen that in 10 a and 10 b the dilution effect has more than counterbalanced the addition of dextrose, so that R_c/Δ is slightly raised. In 15 a and 15 b, however, it is evident that the already low value of R_c/Δ afforded by No. 15 is depressed still further.

In Table X are shown the values given by diabetic urines, not intentionally altered in any way. They had not been heated.

TABLE X.

Diabetic urines.

No.	Dextrose, grams per 100 cc.	Δ	R_c	$\dfrac{R_c}{\Delta}$
33	1·87	1·17°	8·0°	6·84
34	5·62	1·56	10·0	6·41
35	?	1·75	11·0	6·30
36	0·19	1·94	15·3	7·88
37	2·34	2·03	16·0	7·88
38	3·38	2·27	16·5	7·56
			Mean	7·14

Here again the mean value of R_c/Δ is lower than with normal urines, though, owing to the variations of the other constituents of the urine, the decrease is not proportional to the sugar concentration.

We hope to make further experiments with pathological urines, taking into account other abnormal constituents of diabetic specimens.

Summary and Discussion of Results.

The examination of a considerable number of normal urines has shown that the rise in critical solution temperature is roughly eight times as great as the depression of freezing point. This holds good for urines of normal concentrations, but with very dilute urines the critical solution temperature rise is somewhat greater, consequently R_c/Δ has a value greater than eight. This is also the case in specimens which, for any cause, contain an unusually large quantity of salts. On the other hand, the presence of an exceptionally great amount of urea occasions a smaller rise of critical solution temperature; glucose, when present in quantity, has the same effect, but it is far less marked. In the last two cases accordingly the ratio R_c/Δ is somewhat below eight.

This relation between the freezing point depression and the rise in critical solution temperature enables one to transfer the conclusions established by the former method to the latter. Thus A. v. Koranyi [1897, 1899 : Quoted from Casper and Richter] gives $1\cdot3° - 2\cdot3°$ as the usual limits for the freezing point of urine, from a normal kidney under ordinary conditions. Taking $R_c/\Delta = 8$, this range corresponds to a rise in critical solution temperature of $10\cdot4° - 18\cdot4°$.

Lindemann [1899 : Quoted from Casper and Richter] gives a more extended range, $0\cdot9° - 2\cdot7°$, for values of Δ, and states that kidney patients almost always excrete urine for which Δ is less than $1\cdot0°$. Accordingly, taking Lindemann's limit, $0\cdot9°$, and allowing for experimental error and fluctuations due to the composition of the sample, it seems safe to take a rise of $8°$ C. in the critical solution temperature as indicating that the kidney supplying the sample was functioning satisfactorily. It must be remembered, however, that the converse is not always true; thus a specimen giving a rise of $7°$ might really be normal, as, owing to the ingestion of a large amount of liquid, or to the presence of much urea, the value of R_c could very well be lowered.

In the latter limiting case, it would be well to make a freezing point determination or rough urea determination by the hypobromite method, to see if there was an unusual quantity of this substance. Ordinary values of R_c lie between $11°$ and $16°$ C.

In conclusion, we wish to thank Dr J. Timmermans, of Brussels, for putting his extensive knowledge of critical solution point determinations

entirely at our disposal during his visit to Trinity College.   A detailed treatment of the subject will be found in his paper [1907][1].

REFERENCES.

Atkins (1908), *Brit. Med. J.* **1**, 254.
Benedict and Gephart (1908), *J. Amer. Chem. Soc.* **30**, 1760.
Casper and Richter, *Diagnosis of Kidney Disease*, p. 95.
Folin (1912), *J. Biol. Chem.* **11**, 507.
Koranyi (1897), *Zeitsch. klin. Med.*
—— (1899), *Berlin klin. Wochsch.*
Lindemann (1899), *Arch. klin. Med.* **65**.
Philip (1903), *J. Chem. Soc.* **83**, 814.
Schreinemakers (1899), *Zeitsch. physikal. Chem.* **29**, 597.
Timmermans (1907), *Zeitsch. physikal. Chem.* **58**, 129.

[1] See also Findlay, *The Phase Rule*, and Young, *Stoichiometry*.   London, Longmans, Green & Co.

XXIV. QUANTITATIVE RELATIONS IN CAPILLARY ANALYSIS.

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When a dispersed system is allowed to fall on filter- or blotting-paper or to rise up into a strip of such a paper, one observes that the dispersed phase remains behind the medium of dispersion, which is in most cases water. This phenomenon, which involves an increase in the concentration of the dispersed phase in the paper, can be used for the qualitative testing of the nature of the substance as has been shown by the capillary analytical experiments of Goppelsroeder [1909].

Holmgren [1908] tried to establish a formula, which gives a quantitative relation between the extents of the dispersion medium and the dispersed phase. He experimented with diluted acids, especially with hydrochloric acid, by allowing the latter to drop on blotting-paper. Using Congo-red as indicator, he was able to show how far the acid was spread over the paper compared with the water. If a good and homogeneous blotting-paper is used, the figure caused by the diffusion of the drop approaches a circle.

Holmgren supposed that the relation between the water-zone, the largest radius of which may be R, and the inner circle of radius r caused by the acid, is constant for each concentration, so that the quotient of two different concentrations is equal to the quotient of these relations. Therefore, as $[\pi R^2 - \pi r^2]$ is the surface of the water-ring, the following relation exists according to Holmgren:

$$\frac{C}{C_1} = \frac{\dfrac{r^2}{R^2 - r^2}}{\dfrac{r_1^2}{R_1^2 - r_1^2}} \quad \dotfill (1).$$

If we signify by P that concentration for which

$$\frac{r_1^2}{R_1^2 - r_1^2} = 1.$$

the following equation results:

$$C = P \cdot \frac{r^2}{R^2 - r^2} \ldots\ldots\ldots \ldots\ldots\ldots\ldots\ldots(2),$$

or

$$P = C \cdot \frac{R^2 - r^2}{r^2} \ldots\ldots\ldots\ldots\ldots\ldots\ldots(3).$$

If, therefore, the concentration C and the radii of the circles produced on blotting-paper by any drop of the acid are known, P is determined from equation (3). Holmgren supposed P to be constant for the same paper and independent of the concentration. He called P the paper constant and was then able to determine any other concentration by the equation (2), but with the limitation that this method was not to be used except for very dilute solutions between 2·0 and 0·01 %. The results of Goppelsroeder's experiments, which give only the measurements of capillary height in strips of filter-paper, can also be treated by this formula of Holmgren, if instead of the height-measurements their squares are taken.

Skraup [1909, 1 and 2] and his co-workers were able to confirm the applicability of this formula for capillary-analysis. On examining the amount of free hydrochloric acid in the gastric juice by this method, I found [Schmidt, 1913] that the results obtained agreed very closely with those obtained by titration. Later some doubts arose in my mind as to the correctness of Holmgren's formula and occasioned me to undertake the following investigation.

Technique of Experiments.

I experimented exclusively with hydrochloric acid, an exactly adjusted normal solution of which was taken as the basis for the following dilutions between 2·0 and 0·05 %. One drop of these solutions was allowed to fall on blotting-paper from a short distance. Much attention was paid to insure that the drop had always the same volume. With an ordinary pipette it was not possible to get the volumes of the drops as exact as was desirable. I therefore used the dropping surface of Traube's stalagmometer, which gives exactly equal drops, if the temperature be kept constant and the instrument be not allowed to deviate from the vertical position. Blotting-papers are much better fitted for capillary analysis than filter-paper, but they must not be too thick and must have a smooth surface and a most homogeneous structure. I obtained a series of different blotting-papers from the firm of C. Schleicher und Schüll, Düren i. Rheinland. The papers marked Nos. 123

and 117 proved to be specially suitable for my purpose. The papers were stained by floating them rapidly through an alcoholic solution of Congo-red (0·04 %) or methyl-orange (0·1 %). In order to prevent the water from evaporating or at least to reduce the evaporation to a minimum, as soon as the drop was absorbed the paper was placed between two glass plates and a weight laid on the plate to avoid any wrinkling. The plates were then put on a small frame under a glass globe, the interior of which was kept saturated with moisture by means of wetted filter-paper below. To neglect the evaporation leads to false conclusions. The radii were measured in millimetres by the use of transmitted or reflected light, and since the figures of the larger spots are mostly elliptical owing to the direction of the fibres in the structure of the paper, the main axes as well as two oblique axes were measured, so that the given result is the average of the measurement of 4 diameters. The number of figures examined to determine any single point is in most cases at least 4. The measurement took place after exactly one hour, the process having then practically reached a standstill. In reality one may sometimes observe an increase of the radii even after 24 hours, but the amount of it is too insignificant to be noted, especially if only one drop is taken.

Referring to dilutions of acid under 0·1 % the measurement must take place earlier than one hour especially when Congo-red is used, because the blue colour disappears after a certain time, owing to chemical action.

EXPERIMENTS.

The mass of one drop given by a stalagmometer at the temperature of 18–20 is indicated by a. If the drop consisted of distilled water, its weight was found to be 0·1160 gr., and if it consisted of HCl of a concentration between 2·0 and 0·05 %, I found the average weight of a drop 0·116 gr., the differences of the specific gravity in so highly diluted acids being so small that they can be neglected without introducing an error which exceeds the error of observation.

Therefore, putting the volume equal to the weight (in case of dilute HCl) I give a the value of 0·116 gr. Supposing m parts of hydrochloric acid to be present in the drop a, the concentration is given by $C = \frac{m}{a}$ by means of which m is easily calculated.

Under the above described conditions, a series of observations was made, of which the following may be reproduced:

TABLE I.

Paper No. 123. $a = 0.116$ gr.

Indicator:		Congo-red						Methyl-orange			
Number of drops:—		1 a		2 a		3 a		1 a		2 a	
HCl %	m	R	r	R	r	R	r	R	r	R	r
2·0	2·32	23·12	21·87	32	29·37	37·87	34·25	23·12	21·62	29·81	26·43
1·0	1·16	23·5	19·62	32·37	26·5	38·5	31·25	23·37	19·56	31·75	26
0·75	0·87	24	18·37	32·37	24·5	38·7	29·5	23·37	18	32	24·25
0·5	0·58	23·62	16·56	31·87	22·18	37·8	26·5	24	16·87	32·25	22·43
0·4	0·464	23·87	15·75	32·75	21·5	38·2	22·12 (?)	23·62	15·56	32·37	20·75
0·3	0·348	23·62	14·63	32·25	19·68	37·0	23·5	23·62	14·75	31·62	19·56
0·2	0·232	23·37	12·37	31·5	17·12	37·3	20·62	23·62	12·25	32·12	17·12
0·1	0·116	23·5	10	31·62	13·5	37·8	16·62	23·62	10·75	32	13·75
0·05	0·058	23·7	8·62	—	—	—	—	23·43	—	—	—
Means of R		23·48		32·09				23·53		31·74	

TABLE II.

Paper No. 117. $a = 0.116$ gr.   Indicator: Congo-red.

Number of drops:		1 a		2 a		3 a	
HCl %	m	R	r	R	r	R	r
1·0	1·16	26·5	24·25	36·0	32·41	42·25	37·5
0·75	0·87	26·85	22·40	35·75	30·1	42·5	35·5
0·5	0·58	26·68	20·62	35·43	27·75	41·62	33·18
0·4	0·464	26·87	20·5	35·43	27·5	43	33·0
0·3	0·348	25·87	18·85	35·87	25·2	42·37	30·3
0·2	0·232	26·25	16·37	36·0	22·81	42·0	26·75
0·1	0·116	26·25	13·63	35·75	18·5	42·25	22·75
0·05	0·058	26·75	—	—	—	—	—
Means of R		26·50		35·75		42·28	

TABLE III.

Paper No. 123. $a = 0.0311$ gr.

		Indicator:	Congo-red		Methyl-orange	
HCl %	m		R	r	R	r
1·0	0·311		14·75	13·29	—	—
0·5	0·155		15·1	12·5	12·5	9·3
0·4	0·124		14·65	9·4	12·8	8·9
0·3	0·0933		14·84	8·85	12·7	8·25
0·2	0·0622		15·15	7·95	12·8	7
0·182	0·0566		13·3	6·4	12·9	6·36
0·1	0·0311		14·26	6·0	13·1	6
	Means of R		14·6		12·8	

On the assumption that the aggregation of acid in the inner circle is of the nature of an adsorption process, the relationship between the two variables m and r must be expressible by the exponential equation

$$r = \beta m^p.$$

It is highly probable also that the concentration of the acid or that of the hydrogen ions is continuously decreasing from the centre to the periphery of the spot-system in the case of any drop of a certain concentration falling on the paper. I suppose then, that r indicates that point of hydrogen ion concentration which corresponds with the sensibility of the indicator. But owing to the impossibility of proving experimentally the gradual decrease of the hydrogen ion concentration in the paper between the centre and r, my supposition remains an hypothesis. To show how in different concentrations the relation between m and r is expressible by the equation $r = \beta m^{\nu}$, we use the graphic method in constructing the m-r-curve Fig. 1.
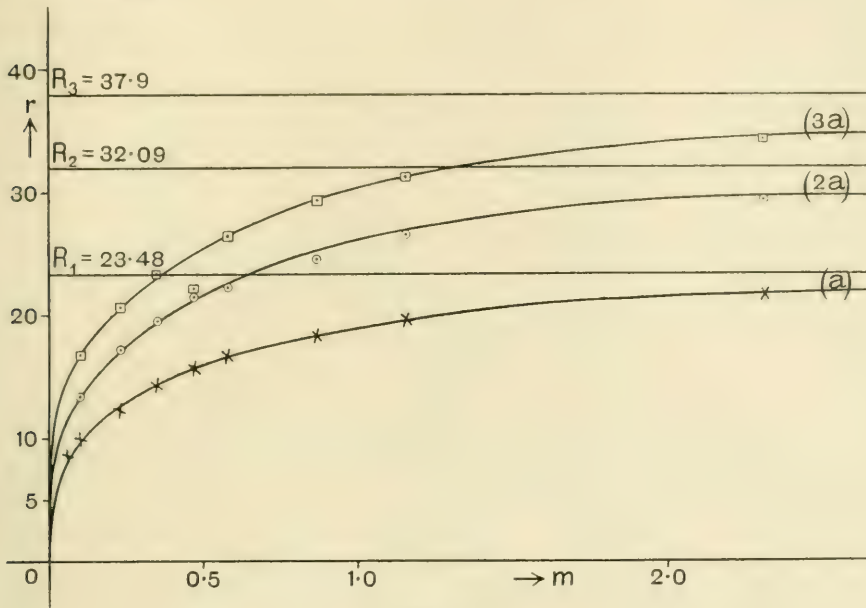
Fig. 1.

The m-r-curve rises very steeply in the beginning and then runs nearly parallel to the m-axis, asymptotically approaching a straight line, of which the equation is $r = R$. The difference $(R - r)$ is identical with the breadth of the water-zone and the latter is of course equal to R in case of distilled water, when m or $C = 0$. $(R - r)$ decreases with increase in the amount of m. Theoretically, the decrease of $(R - r)$ is infinite but in reality m reaches very soon such an amount, that the water-zone is no longer visible. When the latter effect occurs with one drop, the phenomenon can still be obtained by increasing the volume of the drop or augmenting the number of drops, but the errors of observation are also increasing and in such a

degree that for concentrations higher than $2 \cdot 0 \, \%$ the method of Goppelsroeder is preferable, which, as already mentioned, involves the measurement of the capillary height in strips of filter-paper.

That R is in reality the constant, which it seems to be in the experimental data, and that R depends—in the same paper—only upon the volume of the drop and not upon the concentration, will be shown later.

By taking the logs. of the equation $r = \beta \cdot m^p$, we get

$$\log r = p \cdot \log m + \log \beta.$$

This is the equation of a straight line, which is very nearly approached by plotting the values of $\log r$ and $\log m$, as is shown in Fig. 2.
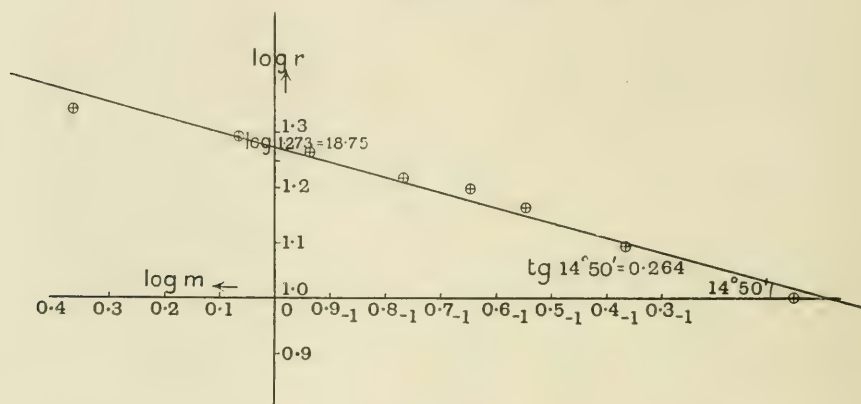
Fig. 2.

By graphic interpolation according to Freundlich's [1907] procedure the values of β and p are found to be

$$p = 0 \cdot 264$$

and

$$\beta = 18 \cdot 75.$$

In the following table the values are calculated by the equation

$$r = 18 \cdot 75 m^{0 \cdot 264} \qquad \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots(4).$$

TABLE IV.

Paper 123. Congo-red. 1 drop ($a = 0 \cdot 116$ gr.). $r = 18 \cdot 75 m^{0 \cdot 264}$.

$C = \%$ HCl	m	r calculated	r observed	Δ calc. − obs.
2·0	2·32	23·41	21·87	− 1·54
1·0	1·16	19·49	19·62	+ 0·13
0·75	0·87	18·08	18·37	+ 0·29
0·5	0·58	16·31	16·56	+ 0·15
0·4	0·464	15·12	15·75	+ 0·63
0·3	0·348	14·19	14·63	+ 0·44
0·2	0·232	12·74	12·37	− 0·37
0·1	0·116	10·61	10	− 0·61
0·05	0·058	8·84	8·62	− 0·22

Although the agreement is fairly good, a further calculation of β and p was made by the following procedure in order to diminish the differences.

HCl %	$100 \times m$	$100 \times r$	$\log m$	$\log r$
2·0	232	2187	2·36549	3·33985
1·0	116	1962	2·06446	3·29248
0·75	87	1837	1·93952	3·26411
0·5	58	1656	1·77085	3·21906
0·4	46·4	1575	1·64652	3·19728
0·3	34·8	1463	1·54158	3·16524
0·2	23·2	1237	1·36549	3·09237
0·1	11·6	1000	1·06446	3
0·05	5·8	862	0·76343	2·93551
		Mean	1·613533	3·167322

Deviations from means :

m_1	r_1	$m_1 \cdot r_1$	$(m_1)^2$
$+0·75195$	$+0·172528$	0·12957	0·56543
$+0·45092$	$+0·125158$	0·056432	0·20333
$+0·32598$	$+0·096788$	0·031551	0·051067
$+0·15731$	$+0·051738$	0·0081424	0·024746
$+0·032987$	$+0·029958$	0·00098825	0·0010881
$-0·071953$	$-0·002082$	0·000119806	0·0051772
$-0·248043$	$-0·054952$	0·013630	0·061523
$-0·549073$	$-0·167322$	0·091871	0·30147
$-0·850103$	$-0·231812$	0·19705	0·72267

$$P = \frac{\Sigma (m_1 \cdot r_1)}{\Sigma (m_1)^2} = \frac{0·529384}{1·9365013} = 0·2733.$$

The value 0·273 for the exponent p gives the following values for

$$\log \beta_1 = \log r - 0·273 \log m.$$

$\log \beta_1$	and for β_1
2·694072	494·4
2·728883	535·6
2·73462	542·7
2·73561	544·0
2·74778	559·4
2·74438	555·1
2·711959	524·3
2·70940	512·1
2·72709	533·4

As the figures for r and m have been multiplied by 100, the values of $\log \beta$ in the equation

$$r = \beta \cdot m^p$$

are calculated from the following equation :

$$\log \beta = \log \beta_1 + 2 \cdot (0·273) - 2.$$

These values of $\log \beta$ are therefore the following :

$\log \beta$	β
1·25007	17·78
1·27488	18·83
1·28062	19·08
1·28161	19·12
1·29378	19·66
1·29038	19·51
1·26559	18·43
1·25540	18·00
1·27309	18·75

Mean of β 18·88

It results therefore for the relation between r and m the equation

$$r = 18\cdot88 \, . \, m^{0\cdot273} \quad\dots\dots\dots\dots\dots\dots\dots(5).$$

Thus substituting the calculated values for β and p the following equation was obtained:

$$r = 18\cdot88 \, . \, m^{0\cdot273},$$

which gives a far better agreement between calculation and observation, as is shown in the following table:

TABLE V.

Paper 123. Indicator: Congo-red. One drop. $a = 0\cdot116$ gr. $r = 18\cdot88 \, . \, m^{0\cdot273}$.

$C = ^0/_0$ HCl	m	r calculated	r observed	Δ
2·0	2·32	23·75	21·87	− 1·88
1·0	1·16	19·66	19·62	− 0·04
0·75	0·87	18·17	18·37	+ 0·2
0·5	0·58	16·34	16·56	+ 0·22
0·4	0·464	15·11	15·75	+ 0·64
0·3	0·348	14·15	14·63	+ 0·48
0·2	0·232	12·67	12·37	− 0·3
0·1	0·116	10·48	10·0	− 0·48
0·05	0·058	8·677	8·62	− 0·05

Idem, but $a = 0\cdot0311$ gr.

1·0	0·311	13·72	13·29	− 0·43
0·5	0·155	11·09	12·5	+ 1·41
0·4	0·124	10·67	9·4	− 1·27
0·3	0·0933	9·880	8·85	− 1·03
0·2	0·0622	8·845	7·95	− 0·89
0·182	0·0566	8·620	6·4	− 2·42
0·1	0·0311	7·320	6	− 1·32

The reason why the second part of the Table V does not show so good an agreement is the inexact measurement of drops by using an ordinary pipette instead of a stalagmometer. By increasing the number of drops, so that instead of a, $2a$ or $3a$ were taken of the same concentration $C = \dfrac{m}{a}$, the radii of the corresponding spots must be

$$r\sqrt{2}, \quad r\sqrt{3},$$

or, generally speaking, if $[na]$ is taken as the dropping mass, the corresponding

radius is $r\sqrt{n}$, r being the radius for a. Therefore, if the radii found for a are supposed to be fixed, the radii for $2a$ or $3a$ can be calculated. The following table gives the results:

TABLE VI.

Paper 123.   Indicator: Congo-red. $a = 0.116$ gr.

Number of drops:	a	$2a$		$3a$	
$C = \%$ HCl	r_1	r_2 calculated	r_2 observed	r_3 calculated	r_3 observed
2·0	21·87	30·83	29·375	37·63	34·25
1·0	19·62	27·66	26·5	33·98	31·25
0·75	18·37	25·90	24·5	31·81	29·5
0·5	16·56	23·34	22·185	28·68	26·5
0·4	15·75	22·20	21·5	27·27	22·125 (?)
0·3	14·63	20·63	19·685	25·33	23·5
0·2	12·37	17·44	17·125	21·42	20·625
0·1	10·0	14·14	13·5	17·32	16·625
0·05	8·62	—	—	—	—

The agreement between the calculated and observed values for r is still fairly good for $2a$. But by increasing the number of drops beyond 2, the experimental error increases, because the figure produced on paper loses the resemblance to a circle, so that the average of r is determined with more arbitrariness. By using the equation (5)

$$r = 18.88 \cdot m^{0.273}$$

for cases where, generally speaking, (na) was taken, m becomes (nm). The values of r_2 for $2m$ and of r_3 for $3m$ are shown in the following table:

TABLE VII.

Paper No. 123.   Indicator: Congo-red. $a = 0.116$ gr. $r\sqrt{n} = 18.88 \, (nm)^{0.273}$.

$C = \%$ HCl	$2m$	log $2m$	r_2 calculated	r_2 observed	Δ observed − calc.	Δ r_2 calculated − $r_2 = r_1\sqrt{2}$
2·0	4·64	0·66652	28·71	29·37	+0·66	−2·12
1·0	2·32	0·36549	23·75	26·5	+3·25	−3·91
0·75	1·74	0·24055	21·96	24·5	+2·54	−3·94
0·5	1·16	0·06446	19·66	22·18	+2·52	−3·68
0·4	0·928	0·96755 − 1	18·38	21·5	+3·12	−3·82
0·3	0·696	0·84261 − 1	17·10	19·68	+2·58	−3·53
0·2	0·464	0·66652 − 1	15·31	17·12	+1·81	−2·13
0·1	0·232	0·36549 − 1	12·67	13·5	+0·83	−1·47

$C = \%$ HCl	$3m$	log $3m$	r_3 calculated	r_3 observed	Δ observed − calc.	Δ r_3 calculated − $r_3 = r_1\sqrt{3}$
2·0	6·96	0·84261	32·06	34·25	+2·09	−5·57
1·0	3·48	0·54158	26·53	31·25	+4·72	−7·45
0·75	2·61	0·41664	24·53	29·5	+4·97	−7·28
0·5	1·74	0·24055	21·96	26·5	+4·54	−6·72
0·4	1·392	0·13364	20·53	22·12 (?)	+1·59 (?)	−6·74
0·3	1·044	0·01870	19·10	23·5	+4·4	−6·23
0·2	0·696	0·84261 − 1	17·10	20·62	+3·52	−4·32
0·1	0·348	0·54158 − 1	14·15	16·62	+2·47	−3·17

The deviations of the calculated figures of r from those obtained by observation are so considerable as to make it clear that the volume used for capillary-analytic purposes must not exceed a certain quantity. But the deviation of the calculated values $r_1 \sqrt{n}$ and $18.88 m^{0.273}$ must be due to the inexactness of the constant in the equation $r = \beta m^\nu$ and to the experimental errors for r_1. In reality the relation

$$r_1 \sqrt{n} = \beta \, (n \, . \, m^\nu) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(6)$$

must exist.

THEORETICAL PART.

On detailed consideration of the processes taking place in the paper, it becomes evident that an increase of concentration of the hydrochloric acid has taken place.

If C signify the original concentration and C_1 the resulting concentration in the coloured spot, the relation between these concentrations may be deduced in the following way. The paper is supposed to be previously stained either with 0.04% alcoholic Congo-red solution, or with 0.1% alcoholic methyl-orange solution, and the staining to be done in the same manner, the paper not being allowed to adsorb much of the dye, a process which is at room temperature only a matter of time, as has been shown by W. M. Bayliss [1906]. Observation shows that stained paper differs slightly in quality from unstained, a fact to which reference will be made later.

Let $[a]$ be the constant quantity of the drop and $[na]$ the quantity of hydrochloric acid of the concentration $C = \frac{m}{a}$, dropping on a piece of blotting-paper which is supposed to be always of the same quality. The quantity $[na]$ of the acid may spread over the paper, so that the acid reaches from the centre to the distance $r \sqrt{n}$, and the largest radius of the water-zone may be $R \sqrt{n}$, if r and R are the corresponding values for $[a]$. In case of another drop-volume, the weight being g', the radius becomes $r \sqrt{\dfrac{g'}{g}}$, g being 0.116 gr.

As the acid constituent of $[na]$ is in the interstices of the paper, the volume $[na]$ must be equal to the volume of the paper, the air being included, minus the specific volume of the paper-material itself in the same space. The volume occupied by $[na]$ is $\pi n R^2$ and the specific volume of the paper-material is its weight divided by its specific gravity s. The weight of a portion of paper of square millimetre surface and thickness δ is indicated by p. Therefore the following equation results for $[na]$:

$$na = \pi n R^2 \delta - \pi n R^2 \frac{p}{s} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots(7),$$

or, if $n = 1$, $\qquad a = R^2 \cdot \pi \left(\delta - \frac{p}{s} \right).$

$\pi \left(\delta - \frac{p}{s} \right)$ represents a constant characteristic for the paper and may be indicated by k, so that

$$k = \pi \cdot \left(\delta - \frac{p}{s} \right)$$

and $\qquad\qquad\qquad a = R^2 \cdot k,$

or $\qquad\qquad\qquad R = \sqrt{\dfrac{a}{k}} \ \dotfill (8).$

That is to say, R depends only upon the variation in quality of the paper and upon the drop-volume which is taken, but it is independent of the concentration C. Therefore R is to be considered as constant for all concentrations, if the same paper and the same drop-volume are used.

The easiest and quickest way to find k is by means of the equation (8), but by weighing the paper and determining its specific gravity and thickness the same value for k will be obtained by the equation $k = \pi \cdot \left(\delta - \frac{p}{s} \right).$

In order to determine the thickness δ of the paper, a certain number of sheets are placed between two glass plates and by pressing them very tightly the measure of the distance may be found, which gives when divided by the number of sheets a rough value of δ. The best manner to determine the specific gravities is the use of a pyknometer. The following data were thus obtained:

Paper No. 123	$\delta = 0{\cdot}17$ mm.
	$p = 0{\cdot}1408$ mgr.
	$s = 1{\cdot}36$
	$k = 0{\cdot}20923$
Paper No. 117	$\delta = 0{\cdot}16$ mm.
	$p = 0{\cdot}118$ mgr.
	$s = 1{\cdot}1$
	$k = 0{\cdot}1651$

When the value of k is found in this way, R can be calculated by the equation (8) as is shown in the following table:

TABLE VIII.

Paper No.	a (mgr.)	R calculated			R observed		
		a	$2a$	$3a$	a	$2a$	$3a$
123	116	23·537	33·28	40·77	23·48	32·09	37·9
117	116	26·49	37·48	45·90	26·5	35·75	42·28
123	13·1	12·165	—	—	12·8 Methyl-orange 14·6 Congo-red	—	

The agreement is very close for a, but here, too, it is apparent that increase of the number of drops increases the inexactness of the observations. If the value of R_1 is fixed, R_2 and R_3 can be calculated as $R_1 \sqrt{2}$ and $R_1 \sqrt{3}$, which give also a fairly good agreement, as the following figures demonstrate:

TABLE IX.

Paper No.	a (mgr.)	R_1	$R_2 = R_1\sqrt{2}$	$R_3 = R_1\sqrt{3}$
123	116	23·5	33·13	40·70
117	116	26·5	37·36	45·89

If $C = \frac{m}{a}$, the quantity na of HCl contains nm parts of it. These nm parts of HCl have been retained by adsorption in the inner circle (r), and if v indicates the volume of its interstices, the following equation must express the resulting concentration C_1:

$$C_1 = \frac{n \cdot m}{v}.$$

I neglect the possibility, that the fibres may swell, supposing that the adsorption process is much sooner finished than the swelling of the fibres.

By analogy with equation (7), v can be expressed by

$$v = \pi n r^2 \delta - \pi n r^2 \cdot \frac{p}{s}.$$

After substituting this value for v, C_1 is given by

$$C_1 = \frac{n \cdot m}{n r^2 \pi \cdot \left(\delta - \frac{p}{s} \right)},$$

or
$$C_1 = \frac{m}{r^2 k} \quad \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots(9).$$

This is the equation giving the resulting concentration in the coloured spot, and if z represents the increase of concentration, so that

$$C_1 = z + C,$$

and one substitutes
$$C_1 = \frac{m}{r^2 \cdot k}$$

and
$$C = \frac{m}{a},$$

the following equation results:

$$\frac{m}{r^2 k} = z + \frac{m}{a},$$

as
$$a = k \cdot R^2,$$

$$z = \frac{m}{r^2 k} - \frac{m}{R^2 k},$$

$$z = \frac{m R^2 - m r^2}{k \cdot R^2 \cdot r^2}.$$

If we substitute $\qquad k \cdot R^2 = a,$

and $\qquad\qquad\qquad\qquad \dfrac{m}{a} = C,$

we find $\qquad\qquad\qquad z = C \cdot \dfrac{R^2 - r^2}{r^2}$ ....(10).

Therefore z is found to be equal to the value of the paper-constant P of Holmgren (3).

Holmgren was of opinion that the relation between the resulting concentration C_1 in the paper and the original concentration C can be expressed by the quotient $\dfrac{R^2}{r^2}$, so that

$$C_1 = \dfrac{R^2 C}{r^2} \qquad\qquad\qquad\qquad (11).$$

This view seems to be correct, for putting

$$C_1 = \frac{R^2 \cdot C}{r^2} = \frac{m}{r^2 k},$$

we find $R^2 \cdot C \cdot k = m$, and substituting $R^2 k = a$, the definition of $C = \dfrac{m}{a}$ results.

When Holmgren eliminated $r^2 = \dfrac{C \cdot R^2}{P + C}$ out of the equation (3) and substituted this value for r^2 in the equation (11), he got

$$C_1 = P + C \qquad\qquad\qquad\qquad (12).$$

Therefore Holmgren's paper-constant corresponds with z in equation (10) representing the increase of concentration by the adsorption, P therefore not being a constant but a function of the concentration in question. The following table shows the values for C_1 calculated by the equation (9), P calculated by the equation (2) and C_1 calculated by formula (12).

TABLE X.

Paper No. 123. Congo-red. One drop ($a = 0.116$ gr.). $k = 0.20923$.

$C = \%\ \mathrm{HCl}$	$C_1 = \dfrac{m}{r^2 \cdot k}$; $\%$	$P = C \dfrac{R^2 - r^2}{r^2}$	$C_1 = P + C$	$P = 0.441$ $C_1 = 0.441 + C$
2·0	2·32	0·305	2·305	2·441
1·0	1·44	0·432	1·432	1·44
0·75	1·23	0·475	1·225	1·19
0·5	0·994	0·505	1·005	0·94
0·4	0·894	0·488	0·888	0·841
0·3	0·777	0·472	0·772	0·741
0·2	0·724	0·520	0·720	0·641
0·1	0·554	0·451	0·551	0·541
0·05	0·373	0·321	0·371	0·491

Mean of $P = 0.441$.

TABLE X (cont.)

Paper No. 117. Congo-red. One drop $(a=0\cdot116\text{ gr.})$. $k=0\cdot1651$.

$C=\%\text{ HCl}$	$C_1=\dfrac{m}{r^2\cdot k}\ (\%)$	$P=C\dfrac{R^2-r^2}{r^2}$	$C_1=P+C$	$P=0\cdot285$ $C_1=0\cdot285+C$
1·0	1·19	0·194	1·194	1·285
0·75	1·05	0·299	1·049	1·035
0·5	0·825	0·325	0·825	0·785
0·4	0·668	0·286	0·686	0·685
0·3	0·593	0·292	0·592	0·585
0·2	0·524	0·323	0·523	0·485
0·1	0·377	0·277	0·377	0·385

We see that the values given by $C_1=\dfrac{m}{r^2 k}$ and $C=P+C$ are almost identical as the theory demands. But if P is supposed to be constant according to Holmgren, and if the average value is taken for P, we find that the values no longer agree so closely. This difference is better demonstrated by the graphic method in constructing the $C\text{-}C_1$-curve (Fig. 3).
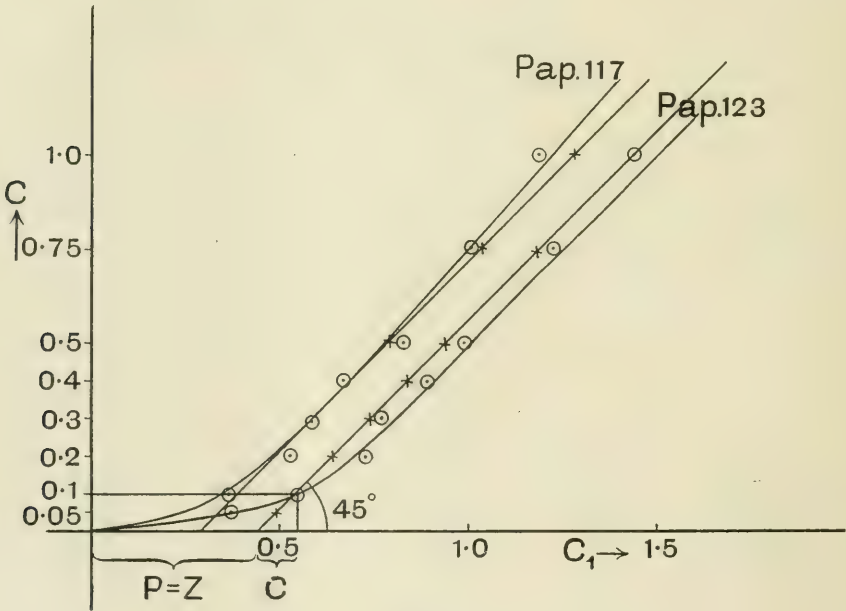
Fig. 3.

It is evident that $C_1=P+C$ represents a straight line, which cuts the C_1-axis at a distance P from the origin and the inclination of which must be 45°.

$C_1 = \frac{m}{r^2 k}$ represents a parabola, by means of which it is possible to demonstrate the fact that for $C = 0$, C_1 must also be 0, a fact which is not expressible by $C_1 = P + C$, P supposed to be constant.

In reality, as P varies with the concentration, the equation $C_1 = P + C$ represents a system of parallel straight lines and the points indicating the relation between C and C_1 follow the course of a parabola cutting this system.

Nevertheless, the disagreement between Holmgren's supposition and the observed values is so slight, that for concentrations not under 0·1 %, his method is for practical purposes very useful on account of its extraordinary facility. To demonstrate the very close agreement of the calculation by Holmgren's formula (2) with the reality I give the following table:

TABLE XI.

Paper 123. $a = 0\cdot116$ gr.

$C = \%$ HCl	$P = 0\cdot441$ (const.) $C = P \dfrac{r^2}{R^2 - r^2}$	$m = {}^{0\cdot273}\!\!\sqrt{\dfrac{r}{18\cdot88}}$ $\dfrac{m}{a} = \%$
2·0	2·87	1·4
1·0	1·02	0·989
0·75	0·69	0·77
0·5	0·43	0·53
0·4	0·36	0·44
0·3	0·28	0·33
0·2	0·16	0·18
0·1	0·097	0·084
0·05	0·068	0·048

With regard to a possible combination of the dye and the acid in the paper, the amount of Congo-red or methyl-orange can be approximately calculated. Bayliss [1906] has shown in regard to Congo-red, that the staining of paper is an adsorption process. The temperature-coefficient of the reaction-velocity is so low, that at room temperature at least 24 hours are required for the attainment of equilibrium. I used a clear looking alcoholic solution of Congo-red (0·04 %) and of methyl-orange (0·1 %) always at room-temperature, and dipped the piece of paper into these solutions only for a few seconds, then allowing the alcohol to drain off and drying the paper at 37°. It can therefore be presumed that the amount of dye adsorbed is very small, and if it be assumed that in so short a time only that amount of Congo-red (or methyl-orange) can be adsorbed which was present in the solution filling the interstices of the paper-fibres, the possible error is certainly very small.

Of an 0·04 % alcoholic Congo-red solution each cmm. contains 4.10^{-7} g. dye.

or in case of 0.1% methyl-orange $0.1 \cdot 10^{-7}$ g. dye. As already shown, the volume of the interstices, which belongs to a surface of πr^2, has been found to be $r^2 \cdot k$. According to Table I r was found $= 10$ mm. in case of an 0.1% HCl, which gives $r^2 \cdot k = 20.9$ or 21 cmm. Therefore the amount of dye adsorbed in these 21 cmm. is in case of Congo-red $84 \cdot 10^{-7}$, and in case of methyl-orange $2.1 \cdot 10^{-7}$. The same consideration gives $2.1 \cdot 10^{-7}$ as the amount of an 0.1% HCl in the same volume of interstices.

These figures are mentioned although I was not able to prove that any chemical action resulting in a perceptible decrease in the amount of free acid took place between dye and acid. No difference in the measurement of the radii of the acid-circles could be observed on varying the concentration of the indicator-solution. Holmgren, who compared the effect of 1.0 and 0.1% solutions of Congo-red in water on the extent of the surface produced by allowing a drop of acid to fall on paper under similar conditions, thought it also very improbable that the amount of dye by itself plays any important part.

But if the effect of adsorption in unstained paper is compared with that obtained in stained paper, a difference can be observed.

Experimenting under the conditions described in the introduction, one observes in unstained paper that the extent of the water-zone exceeds by 2.5 mm. that found in paper stained by the alcoholic indicator-solutions. This difference in the extent of the water-zone is constant, when the concentration of the acid employed varies, a fact which agrees with the circumstance that R is independent of the concentration.

The extent (r) of the acid-spot is also less in stained paper than in unstained, but according to observation this difference increases the more the acid has been diluted, which favours the idea that a chemical action occurs between acid and indicator.

The weight of about 500 gr., which I used to prevent the paper from wrinkling, influences the extent of the water-zone, but the enlargement caused by the pressure is very insignificant and does not exceed 0.1 mm.

In studying the influence of pure alcohol on the paper regarding the adsorption of hydrochloric acid and the capillary extension of water, experiments showed that pure alcohol has an inhibiting action on water as well as on diluted acid.

I may suggest that the fibres of the paper when treated by alcohol, whether pure or combined with dye, are inhibited or prevented from swelling by water, and thus the interstices become larger, which would explain the observation that the radii become smaller.

To apply these considerations to the method which involves the measurement of the capillary height in strips of paper the different volume of liquid must be taken into account. Skraup and his co-workers [1910] were able to show that the amount of water raised in strips of filter-paper is different in different parts of the strip, decreasing in a hyperbolic manner [p. 887]. Whether a similar decrease of the amount of water occurs in the case of a drop producing a circle on the filter-paper is not proved, and I think it very improbable, believing that this phenomenon depends upon gravitation and therefore upon the inclination of the strip, the influence of which is shown by Goppelsroeder [1909] in regard to the capillary height.

I found also in strips of paper that the fact whether they are untreated, stained by alcoholic Congo-red solutions or only treated by pure alcohol, must be taken in account. Thus under the same experimental conditions distilled water rises quicker in the unstained paper and the contrary takes place in case of a dilute hydrochloric acid.

The following table contains measurements of capillary heights in mm., the time having been constant for each experiment, but I could not find any satisfactory explanation of the phenomenon.

| | | Paper 123 untreated | Treated by | |
			Pure alcohol	Congo-red
Diluted HCl	Water	59	71	71
	Acid	50	59	59
Distilled water		85	105	77
		66	78	62
		76	89	69

Holmgren [1908] found that the relation between the capillary heights of the acid and the water increases according to the concentration, and that this relation is constant for the same concentration; Skraup and his co-workers [1910] found for all acids (with a few exceptions) that the stronger the acid the higher is the degree of adsorption and vice versa.

I hope later to be able to show how the mathematical considerations described in this paper can be applied also to Goppelsroeder's and Skraup's experimental data obtained by the measurement of the capillary height. But I am conscious of the fact that these formulae are still far from being able to describe all the possibilities in such a complex phenomenon as the adsorption of acids by paper.

Conclusions.

1. Diluted acids produce a ring system when dropped on blotting-paper, the acid remaining behind the water.

2. The radius r of the coloured circle produced by the acid in the paper is connected with the concentration C by an exponential equation of the form $r = \beta \cdot C^n$.

3. The radius R of the water-zone is independent of the concentration and can be determined by the equation $R = \sqrt{\frac{a}{k}}$, a being the volume of the acid drop in question and k being a constant which depends upon the quality of the [stained] paper.

4. The adsorption causes an increase of concentration. The final concentration is found to be a parabolic function of r.

5. The increment of concentration varies with the initial concentration.

6. Holmgren's calculation, which assumes that the increment of concentration is a constant dependent only upon the quality of the paper, is theoretically incorrect, but it has been shown that it may be useful for practical purposes.

REFERENCES.

Bayliss (1906), *Biochem. J.* 1, 175.
Freundlich (1907), *Zeitsch. physikal. Chem.* 57, 391.
Goppelsroeder (1909), *Zeitsch. Chem. Ind. Kolloide*, 4, 23 (where previous papers by the same author are quoted).
Holmgren, J. (1908), *Biochem. Zeitsch.* 14, 181.
—— (1909), *Zeitsch. Chem. Ind. Kolloide*, 4, 219.
—— (1912), *Arch. Verdauungskrankheiten*, 17, Ergänzungsheft 57.
Schmidt, H. (1913), *Deutsch. med. Wochensch.* Nr. 8, 358.
Skraup (1909, 1), *Sitzungsber. K. Akad. Wiss. Wien. Math.-naturwiss. Kl.* 118, Abt. II b, 459, 559.
—— (1909, 2), *Monatsh.* 30, 675, 773; 31, 565, 873.
——, Krause, v. Biehler (1910), *Sitzungsber. K. Akad. Wiss. Wien. Math.-naturwiss. Kl.* 119, Abt. II b, 565.
——, v. Biehler, Lang, Philippi, Priglinger (1910), *Sitzungsber. K. Akad. Wiss. Wien*, 119, Abt. II b, 873 (887).

XXV. A NOTE ON THE HOPKINS AND COLE MODIFICATION OF THE ADAMKIEWICZ TEST FOR PROTEIN.

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INTRODUCTION.

The following brief piece of research is the outcome of an unexpected failure in class-work to obtain the well-known reaction with "reduced oxalic" acid [Hopkins and Cole, 1901, 2] protein and concentrated sulphuric acid. In the previous experience of the writer at Cambridge and elsewhere, the test had never failed, and a high estimate of its reliability had been formed, so that this case, when nothing but a yellow to a yellow-brown ring was obtained, needed investigation.

It was soon found that the failure was due to an accidental admixture of the bench sulphuric acid with nitric acid, but during, and arising out of, the search for the cause, the phenomena recorded below were observed.

It became obvious that three disturbing factors were involved: (*a*) the presence of small quantities of impurities in the sulphuric acid, (*b*) the absence of traces of impurities, and (*c*) physical factors influencing the rise and fall of temperature of the reacting fluids. These have been investigated in turn.

(1) *The presence of impurities in supraminimal amounts.*

The cause of the trouble recorded above was the presence in the sulphuric acid of nitric acid.

The test, as has been pointed out by Salkowski [1888] and Hopkins and Cole [1901, 1], and confirmed by Rosenheim [1906], is spoilt by oxidising agents. This occurs at small concentrations, especially if, as a criterion, rather than the formation of a ring, the colour of the resultant mixture be

taken[1]. Thus a final concentration of 1 part by volume of hydrogen peroxide (10 vols. comm.) in 50, of 1 part by weight of ferric chloride in 1000, of 1 part by weight of potassium chlorate in 25,000, of 1 part by volume of concentrated nitric acid in 25,000 or of 1 part by weight of sodium nitrite in 50,000 parts by volume prevents the formation of a violet or purple coloration.

In all cases except one (nitric acid) a yellow ring is formed, which, on shaking the tube gently from side to side, spreads throughout the mixture. With nitric acid there appears a purple ring above the yellow ring at the junction of the fluids. When the test tube is shaken the purple ring moves up the test tube, is overtaken by the yellow coloration and is destroyed. In no case is there charring.

For laboratory practice, therefore, if, instead of a lilac, mauve or violet coloration, a yellow, a red, a red-purple or a brown ring is formed, it is probably due to contamination of the sulphuric acid with oxidising agents. Commercial sulphuric acid, to judge by the samples investigated, never has enough oxidising agents present to prevent the reaction.

(2)   *The influence of oxidising agents in minimal amounts*

In the course of a large number of experiments it has become clear that Rosenheim's statement that an oxidising agent is necessary to the reaction is justified. The observations on which this is based are as follows (for experimental details consult Appendix, § 2):

(a)   Some very pure samples of sulphuric acid (supplied by Messrs Evans, Lescher and Webb, Messrs Baird and Tatlock, Liverpool, and Messrs Towers and Sons, Widnes and Liverpool) give with freshly made or with old "reduced oxalic," whether strong or weak, or with Benedict's magnesium-reduced oxalic [1909] a lilac or lilac-mauve colour instead of the typical violet (see Appendix, § 2, Exp. LVIII etc.). But if traces of oxidising agents are added to the "reduced oxalic" the blue-violet or violet develops at once (Appendix, § 2). The optimal concentration is, for hydrogen peroxide (10 vols. comm.) *circa* 1 part by volume in 1000 of the resulting mixture, for ferric chloride 1 part by weight in 20,000 and for sodium nitrite 1 part in 375,000.

(b)   Subsequent addition of oxidising agents after the lilac or lilac-mauve coloration with pure sulphuric has developed converts this colour to a blue-violet of great density and beauty (Appendix, § 1, Exps. LII—LIV)[2]. This

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[1] For experimental details see Appendix, § 1, Exps. I, II, XXX, LII—LV.

[2] The paradox of the greater stability of the colour when once formed towards oxidising reagents is simply explained as the decrease in velocity of a chemical reaction concurrent with fall of temperature.

does not always take place if the reacting fluids have cooled. But in this case, warming, with previous or subsequent addition of an oxidising agent, yields the typical blue-violet (Appendix, § 3 and § 6). Continued heating alters the test through a series of colours typically the result of the addition of increasingly greater quantities of oxidising agents to the "reduced oxalic" (Appendix. Compare § 3, with § 2 Exp. LIX and § 4 Exp. LXIV). Obviously change in colour is a sign of advancing oxidation.

(c) Commercial sulphuric acid will withstand the addition of less oxidising agent than will pure sulphuric acid if the test is to be typical. The reaction is not improved but spoilt by the addition of even small traces (Appendix, § 4, Exp. XXXVI, etc.).

(d) Commercial sulphuric acid will withstand the addition of more formaldehyde than will pure sulphuric acid (Appendix, § 4, Exp. XLI and XLII).

(e) If the violet fluid resultant from the use of commercial sulphuric acid be boiled, the colour changes seen in the parallel example quoted in section (b) above are imitated with exactitude (Appendix, § 4, Exp. LXIV). Oxidation by means of the oxidising agents present in the commercial sulphuric acid is proceeding.

These five observations accord well with Rosenheim's theory that oxidising agents are necessary to the reaction. They suggest that a coloured substance arises from the oxidation of a tryptophane-formaldehyde product formed from the protein and that further oxidation of this blue-violet substance modifies this colour and ultimately destroys it. We may assume that this reaction proceeds at a velocity very much greater at high temperatures than at lower temperatures and that, in the optimal conditions of the test, the cooling is rapid enough to prevent the oxidation going too far.

That there is a change proceeding at a definite rate may be seen by carrying out the test under conditions in which a high temperature is not attained, or if attained, is rapidly reduced. For instance the initial colour may be profoundly modified in a very wide tube, or by cooling the test tube under the tap. A rose colour at first develops, but in the first case after the test tube has stood some time the colour has deepened to mauve and after a day to blue-violet. It is doubtful if this be due to the oxygen of the air, for no observable difference was found when the test was carried out under an atmosphere of carbon dioxide.

To the question whether a balance of formaldehyde and oxidising agents is necessary [see Rosenheim, 1906] (another case of the two classical antagonistic principles) my experiments give no certain answer. If these be

both in the "reduced oxalic" and in optimal equipoise, then reduction of the strength of the "reduced oxalic" should render the equipoise more susceptible to tilting agencies. Oxidisers should have more effect in the presence of weak "reduced oxalic" than of strong. There is no evidence to offer that this is the case (App. § 5, Summary of Exps. XVIII to XXXV).

(3) The influence of physical conditions.

It was thought that physical conditions, such as the size and shape of the test tube, area of reacting fluids compared with their volume, and rapidity with which the fluids reached their maximal temperature and subsequently cooled would affect the results. This is indeed so but, except in the extreme cases given above, is not of much importance. Thick test tubes give a lighter result than thin; very narrow than normal size; and very large tubes with inadequate amounts of fluid than normal tubes or large tubes with adequate amounts. Cooling under the tap produces a rose colour shot with green which subsequently, unless oxidising agents are added, does not darken much. In three days it is lilac in colour (see App. § 6).

One reason for the different colours observed when different volumes of the reagents are used is to be found in the dichromatism of the coloured substance. It is lilac in thin layers or great dilutions, and bluer in thicker layers and greater concentrations. This, of course, has its parallel in its absorption band in the spectrum. A mixture that was lilac showed an absorption band from λ 592 to λ 547, on adding a drop of ferric chloride the band stretched from λ 646 to λ 511, diluting the mixture with pure sulphuric acid brought the band to its original dimensions and the mixture to its original colour. On warming the diluted mixture, with consequent alteration in colour to purple, the band stretched from λ 610 to λ 531, and the blue and violet were reduced in intensity. This band is identical with one described by Hopkins and Cole [1901, 1].

Finally, the different colours observed with weaker solutions of protein are due to the oxidation of the chromogen beyond the optimum. Weaker solutions give with either pure or commercial sulphuric acid results that are redder in tint than the controls. This is not dichromatism, for dilution with sulphuric acid leaves the control many degrees bluer than the others. The relation of this phenomenon to the others given above is clear if we assume progressive oxidation.

SUMMARY AND CONCLUSIONS.

(1) The modification of the Adamkiewicz reaction introduced by Hopkins and Cole fails in the presence of small amounts of oxidising agents.

(2) But traces of these improve the reaction when carried out with "pure" sulphuric acid.

(3) Probably, therefore, the result with "commercial" sulphuric acid is due to the presence of contained oxidising agents.

(4) Whether the trace of oxidising agent which gives the lilac to mauve tint with "pure" sulphuric acid is in the reduced oxalic or in the sulphuric acid cannot be settled. In view of Miss Homer's work [1911] it is probably in the sulphuric acid.

(5) The resultant coloration of the mixture depends on the extent of a combination of a chromogen with oxygen. This combination has a definite rate of reaction which is naturally more rapid at high temperature and at greater concentrations of the oxidising agent.

(6) It is therefore best to keep the volumes and percentage compositions of the reacting fluids as constant in proportion as possible and their absolute measure should depend on the size of the test-tube[1].

(7) The following method of carrying out the test yields excellent results: 1 c.c. of strong "reduced oxalic" acid (Benedict's "reduced oxalic" will do just as well) is mixed with 1 c.c. of 1 % Witte's peptone, and then 2 c.c. of sulphuric acid are run down the side of the test-tube which is held as near horizontal as convenient. The fluids are then rapidly mixed by shaking from side to side with the test-tube vertical. The quantities given are for a test-tube 1·5 cm. in diameter (the usual laboratory size). Subsequent addition of one drop of 1 % FeCl$_3$ solution usually increases the density and the blueness of the coloration.

The quantities given may be varied greatly and yet a good coloration be obtained. 1 c.c. or 5 c.c. sulphuric acid to 2 c.c. of the mixture yield an unmistakable result and the quantities may be guessed and not measured. More sulphuric acid gives a bluer result than less.

[1] I can see no reason for abandoning the excellent reagent known as "reduced oxalic" acid in favour of formaldehyde and doctored sulphuric acid. As long as the test is performed under conditions that lead to a maximal development of heat, all samples of sulphuric acid give a reasonable coloration. It is only when the reacting fluids are cooled or contaminated that an imperfect result is obtained; whereas the balance of oxidising agent and formaldehyde is easily tilted in an adverse direction, especially in the hands of students.

"Reduced oxalic" acid is a reagent easily made, particularly now that magnesium powder is a requisite in the biochemical laboratory. It does not diminish in strength even when more than a year old.

.The details of the experiments on which the statements above made are based are relegated to an appendix.

I have to thank Messrs Hopkins and Cole for reading through the MS. of this paper and for suggestions they have made. Their explanation of the phenomena recorded must be left to a future communication from them.

APPENDIX.

1. *Experiments on the destructive effect of oxidising agents.*

In most of the experiments performed the production of a coloration was taken as a criterion and not the formation of a ring. In the experiments based on the plan of Exp. XXX (*v. inf.*)—the majority—all the test tubes were prepared before the shaking of the contents took place. This naturally does not give a maximal rise of temperature. The first test tube has to wait a minute or so before the fluids it contains are mixed and the heat evolved by the natural admixture at the junction of the fluids is to some extent lost. This exaggerates the influence of oxidising agents as adjuvants of the reaction, and decreases the influence as deterrents.

Exp. I. Equal parts stock solution of "reduced oxalic" undiluted and (*circa*) 1 % acid albumin mixed.

Mixture	Pure sulphuric	Oxidiser	Remarks
25 c.c.	25 c.c.	1 drop conc. HNO_3	Yellow colour, faint effervescence.
25 c.c.	25 c.c.	Small amt. $FeCl_3$	Normal violet, disappeared on adding excess $FeCl_3$.
25 c.c.	25 c.c.	1 c.c. 10 vols. H_2O_2	Light yellow coloration.

Exp. II. Same mixture as above.

Mixture.	Comm. sulph.	Oxidiser	Remarks
2 c.c.	2 c.c.	Nil	Violet ring.
2 c.c.	2 c.c.	2 drops 0·01 % HNO_3	Yellow ring.
2 c.c.	2 c.c.	1 drop ,, ,,	Faint violet above, yellow ring below.
2 c.c.	2 c.c.	(1 drop water)	Violet ring.

Exp. XXX. Mixture 1 % peptone and strong "reduced oxalic." Equal parts.

Mixture	H_2O_2 (10 vols.)	H_2O	Pure sulphuric	Remarks	Coloration
2 c.c.	0 drops	5 drops	2 c.c.	Ring not clear.	Lilac-mauve.
2 c.c.	1 ,,	4 ,,	2 c.c.	Good ring.	Clear violet.
2 c.c.	2 ,,	3 ,,	2 c.c.	Good ring.	Purple, then rose.
2 c.c.	3 ,,	2 ,,	2 c.c.	Vanishing ring.	Light red.
2 c.c.	4 ,,	1 ,,	2 c.c.	Vanishing ring.	Yellow.
2 c.c.	5 ,,	0 ,,	2 c.c.	Vanishing ring.	Straw colour.

Throughout the experiments based on this plan the same pipettes were kept for each reagent. The drops were frequently estimated and their

average volume was 0·04 c.c. Of course in the measurement of the above volumes, whether large or small, no great accuracy was obtained. A 1 c.c. pipette has a high percentage error (*circa* 5 %).

Exp. LII. NaNO$_2$. *Same mixture as in Exp. XXX.* In the experiments following, not the formation of a ring, but the final coloration is used as a criterion.

Mixture	0·1 % NaNO$_2$	H$_2$O	Pure sulphuric	Remarks
2 c.c.	0 drops	5 drops	2 c.c.	Lilac coloration (deep blue-violet on adding drop of NaNO$_2$).
2 c.c.	1 ,,	4 ,,	2 c.c.	Dense purple coloration.
2 c.c.	2 ,,	3 ,,	2 c.c.	Red coloration.
2 c.c.	3 ,,	2 ,,	2 c.c.	Orange coloration.
2 c.c.	4 ,,	1 ,,	2 c.c.	Yellow coloration.
2 c.c.	5 ,,	0 ,,	2 c.c.	Yellow coloration.

Exp. LIII. FeCl$_3$.

Mixture	4 % FeCl$_3$	H$_2$O	Pure sulphuric	Remarks
2 c.c.	0 drops	5 drops	2 c.c.	Lilac (dense blue on adding 1 drop FeCl$_3$).
2 c.c.	1 ,,	4 ,,	2 c.c.	Purple coloration.
2 c.c.	2 ,,	3 ,,	2 c.c.	Claret ,,
2 c.c.	3 ,,	2 ,,	2 c.c.	Red-brown ,,
2 c.c.	4 ,,	1 ,,	2 c.c.	Brown ,,
2 c.c.	5 ,,	0 ,,	2 c.c.	,, ,,

Exp. LIV. KClO$_3$.

Mixture	0·2 % KClO$_3$	H$_2$O	Pure sulphuric	Remarks
2 c.c.	0 drops	5 drops	2 c.c.	Lilac coloration (purple on addition of 1 drop KClO$_3$).
2 c.c.	1 ,,	4 ,,	2 c.c.	Purple coloration.
2 c.c.	2 ,,	3 ,,	2 c.c.	Yellow ,,
2 c.c.	3 ,,	2 ,,	2 c.c.	,, ,,

Exp. LV. HNO$_3$.

Mixture	HNO$_3$ 2 vols. per 1000	H$_2$O	Pure sulphuric	Remarks
2 c.c.	0 drops	5 drops	2 c.c.	Lilac (no deepening on adding HNO$_3$).
2 c.c.	1 ,,	4 ,,	2 c.c.	Purple.
2 c.c.	2 ,,	3 ,,	2 c.c.	Red-brown.
2 c.c.	3 ,,	2 ,,	2 c.c.	Yellow.
2 c.c.	4 ,,	1 ,,	2 c.c.	Yellow.
2 c.c.	5 ,,	0 ,,	2 c.c.	Yellow.

In this experiment a purple ring was formed above the junction of fluids with a yellow ring beneath. On gently shaking, the above colours were produced.

A number of other experiments on similar lines were performed with similar results.

2. *Experiments on the beneficial effect of traces of oxidising agents.*
(See also Experiments immediately preceding.)

Exp. VIII.   Peptone mixture.

Mixture	10% H_2O_2 (10 vols.)	H_2O	Pure sulphuric	Remarks
(a) 2 c.c.	0·0 c.c.	1·0 c.c.	2 c.c.	Ring obtained in all. On shaking,
2 c.c.	0·0 c.c.	1·0 c.c.	2 c.c.	(a) (i) and (ii) were the most
(b) 2 c.c.	0·5 c.c.	0·5 c.c.	2 c.c.	violet, (b) the deepest and (c) the
2 c.c.	0·5 c.c.	0·5 c.c.	2 c.c.	weakest. After two hours (b) was
(c) 2 c.c.	1·0 c.c.	0·0 c.c.	2 c.c.	nearly bleached and (c) yellow.
2 c.c.	1·0 c.c.	0·0 c.c.	2 c.c.	

Exp. IX.

Mixture		H_2O	Pure sulphuric	Remarks
2 c.c.	0 drops	5 drops	2 c.c.	Blue-violet coloration.
2 c.c.	1 ,,	4 ,,	2 c.c.	Dense violet, showed up most rapidly.
2 c.c.	2 ,,	3 ,,	2 c.c.	Purple.
2 c.c.	3 ,,	2 ,,	2 c.c.	Purple.
2 c.c.	4 ,,	1 ,,	2 c.c.	Red-purple.
2 c.c.	5 ,,	0 ,,	2 c.c.	Red-purple.

A number of other experiments with different concentrations of H_2O_2 gave similar results. Exp. XXI is quoted. Strong reduced oxalic diluted five times and then mixed with an equal volume of 1% Witte's peptone.

Exp. XXI.

Mixture	4% H_2O_2 (10 vols.)	H_2O	Pure sulphuric	Remarks
2 c.c.	0 drops	5 drops	2 c.c.	Pale mauve.
2 c.c.	1 ,,	4 ,,	2 c.c.	Violet-purple. Three times as dense.
2 c.c.	2 ,,	3 ,,	2 c.c.	Purple.
2 c.c.	3 ,,	2 ,,	2 c.c.	Rose-purple.
2 c.c.	4 ,,	1 ,,	2 c.c.	Purple-rose.
2 c.c.	5 ,,	0 ,,	2 c.c.	,, ,,

Exp. LVIII.   Mixture: 1 part freshly made reduced saturated oxalic and 1 part 1% Witte's peptone.

Mixture	$0·5 \%$ $FeCl_3$	H_2O	Pure sulphuric	Remarks
2 c.c.	0 drops	5 drops	2 c.c.	Lilac. (Deepened to royal blue on subsequent addition of $FeCl_3$).
2 c.c.	1 ,,	4 ,,	2 c.c.	Deep violet.
2 c.c.	2 ,,	3 ,,	2 c.c.	Clear violet.
2 c.c.	3 ,,	2 ,,	2 c.c.	Purple-violet.
2 c.c.	4 ,,	1 ,,	2 c.c.	Purple.
2 c.c.	5 ,,	0 ,,	2 c.c.	Red-purple.

Exp. LIX.   Mixture as above.

Mixture	$0·04 \%$ $NaNO_2$	H_2O	Pure sulphuric	Remarks
2 c.c.	0 drops	5 drops	2 c.c.	Lilac (deepening remarkably on addition of $NaNO_2$ solution).
2 c.c.	1 ,,	4 ,,	2 c.c.	Violet.
2 c.c.	2 ,,	3 ,,	2 c.c.	Purple-violet (strongest at first, bleaching later).
2 c.c.	3 ,,	2 ,,	2 c.c.	Purple.
2 c.c.	4 ,,	1 ,,	2 c.c.	Brown-purple.
2 c.c.	5 ,,	0 ,,	2 c.c.	Purple-brown.

Very many other experiments were performed, some of which are quoted in other sections, with the same results.

3. *Experiments on the subsequent addition of oxidising agents.*

As will be seen in a number of the above experiments, addition of oxidising agents after the lilac coloration was formed produced a deep blue coloration. In some earlier experiments on cooling the fluids while the mixture was being made, it was noticed that the cold mixture of sulphuric acid reduced oxalic and peptone did not turn blue on the addition of ferric chloride, but after the addition warming gave the intense blue coloration. This on standing turned violet. Other tests showed that rewarming the mixture and then adding ferric chloride produced the same result, and that further warming, or rather boiling, made the colour proceed through violet, purple, to red and ultimately bleached it almost completely. Further notes will be found on similar results obtained in the experiments on the effect of cooling the test tube while the sulphuric and the other reagents are mixing (App. § 6).

4. *Comparison between pure and commercial sulphuric acid.*

Exps. XLI and XLII.

Mixture	0·04 % form- aldehyde	H$_2$O	2 c.c. Commercial sulphuric	2 c.c. Pure sulphuric
2 c.c.	0 drops	5 drops	Violet	Lilac-mauve (gives blue ring on running in H$_2$O$_2$).
2 c.c.	1 ,,	4 ,,	Dense violet	Olive green.
2 c.c.	2 ,,	3 ,,	Brown-violet	,, ,,
2 c.c.	3 ,,	2 ,,	Brown-purple	,, ,,
2 c.c.	4 ,,	1 ,,	Purple-brown	,, ,,
2 c.c.	5 ,,	0 ,,	Light purple-brown	,, ,,

Exp. LXIV. Experiments on same plan as above made with different samples of acid.

B. & T. commercial sulphuric		Purple-violet	On heating passed through purple to purple-red and brown.
T.	,, ,,	Purple-violet	
E. L. W.	,, ,,	Purple-violet	
T.	pure ,,	Lilac	On heating slowly altered to purple-brown obtained in XLI, 6.
E. L. W.	,, ,,	Bluish-lilac	

Exp. LXI. When various samples of acids, commercial and pure, were used, but to 2 c.c. of mixture and 4 drops of H$_2$O in each case 1 drop of 0·04 % NaNO$_2$ was added, the following results were obtained :

Sulphuric		Remarks
B. & T. pure,	2 c.c.	Blue-violet.
B. & T. comm.,	,,	Purple.
T. pure,	,,	Blue-violet.
T. comm.,	,,	Purple.
E. L. W. pure,	,,	Blue-violet.
E. L. W. comm.	,,	Purple.

Exps. XXXVI to XL and LX with varying strengths oxidising agents added showed that the commercial sulphuric used had the optimal amount of oxidising agents present. Compare with tests in §§ 2 and 3. As an example XXXVIII is quoted.

Mixture	2% 10 vols. H_2O_2	H_2O	Commercial sulphate	Coloration
2 c.c.	0 drops	5 drops	2 c.c.	Violet (densest).
2 c.c.	1 ,,	4 ,,	2 c.c.	Purple-violet.
2 c.c.	2 ,,	3 ,,	2 c.c.	Purple.
2 c.c.	3 ,,	2 ,,	2 c.c.	Red-purple.
2 c.c.	4 ,,	1 ,,	2 c.c.	Purple-red.
2 c.c.	5 ,,	0 ,,	2 c.c.	Red-brown.

5. *Experiments on the supposed antagonism between formaldehyde and oxidising agents and on the occurrence of the latter in reduced oxalic acid.*

"weak" Hopkins' reagent = reduced saturated oxalic diluted 5 times.
"normal" ,, ,, = ,, ,, ,, ,, $2\frac{1}{2}$,,
"strong" ,, ,, = ,, ,, ,, undiluted.

The experiments were carried out as usual, six tests in each, with increasing strengths and amounts of hydrogen peroxide; thus:

Exp. XXXIII. Mixture of equal parts of strong Hopkins' reagent and 1% peptone.

	Mixture	2% 10 vols. H_2O_2	H_2O	H_2SO_4	Coloration
(a)	2 c.c.	0 drops	5 drops	2 c.c.	Lilac-mauve.
(b)	2 c.c.	1 ,,	4 ,,	2 c.c.	Violet-mauve.
(c)	2 c.c.	2 ,,	3 ,,	2 c.c.	Violet⎫ densest.
(d)	2 c.c.	3 ,,	2 ,,	2 c.c.	Violet⎭
(e)	2 c.c.	4 ,,	1 ,,	2 c.c.	Purple-violet.
(f)	2 c.c.	5 ,,	0 ,,	2 c.c.	Purple.

Summarising the results it was found:

10 vols. H_2O_2	Densest colour		
	Strong reagent	Normal reagent	Weak reagent
10%	(b) (violet)	(b)	(b)
4	(b) & (c) (purple-violet)	(b)	(b)
2%	(c) & (d) (violet)	(d) & (e)	(c)
$1\cdot3\%$	(d) (violet)	(d)	(e)
1%	(b) to (d) (much the same colours)	—	(f)

No evidence is seen of oxidising agents deflecting the optimum towards nil concentration when weak Hopkins' reagent is used. This should be the case if oxidising agents are in the reduced oxalic.

6. Experiments on effect of preventing, or of accelerating, rise to maximal temperature.

Exp. LXV.

Mixture	Pure sulphuric	Procedure	Coloration
2 c.c.	2 c.c.	Shaken under cold tap	Pale rose with green fluorescence.
2 c.c.	2 c.c.	,, ,, warm tap (45° C. circ.)	Rose.
2 c.c.	2 c.c.	Shaken in air	Lilac-mauve.
2 c.c.	2 c.c.	,, ,, Bunsen flame	Violet-mauve, changing to brown-violet.

Repeated first experiment left to stand... ...			Mauve after three days.
,, ,, ,,	added 1 drop 4 %/₀ FeCl₂		Pale rose slowly alters to deep rose, lilac-mauve and on gentle warming to violet.
,, ,, ,,	very thoroughly cooled, added 1 drop 4 % FeCl₃		Slowly darkening in about an hour to lilac-mauve.

7. Experiment on dilution of peptone.

	Peptone	Strong Hopkins' reagent	Sulphuric	Coloration.
(a)	1 c.c. (1 %/₀)	1 c.c.	2 c.c. (pure)	Lilac-mauve.
(b)	1 c.c. ,,	1 c.c.	2 c.c. (comm.)	Violet-purple.
(c)	1 c.c. (0·2 %/₀)	1 c.c.	2 c.c. (pure)	Pale lilac-violet.
(d)	1 c.c. ,,	1 c.c.	2 c.c. (comm.)	Pale purple.
(e)	1 c.c. (0·1 %/₀)	1 c.c.	2 c.c. (pure)	Very pale lilac-purple.
(f)	1 c.c. ,,	1 c.c.	2 c.c. (comm.)	Light rose-purple.

When (a) and (b) are diluted with pure sulphuric the result is bluer than any parallel experiment with weaker peptone. Addition of commercial sulphuric acid to (b) produced a redder colour than addition of pure sulphuric acid to (b). These observations accord with the theory of progressive oxidation of a chromogenic substance by oxidising agents in the sulphuric acid.

REFERENCES.

Benedict (1909), *J. Biol. Chem.* **6**, 51.
Homer (1911), *Proc. Camb. Phil. Soc.* **16**, 405.
Hopkins and Cole (1901, 1), *Proc. Roy. Soc.* **68**, 21.
—— (1901, 2), *Journ. Phys.* **27**, 418.
Rosenheim (1906), *Biochem. J.* **1**, 233.
Salkowski (1888), *Zeitsch. physiol. Chem.* **12**, 211.

XXVI. THE USE OF LITMUS PAPER AS A QUANTITATIVE INDICATOR OF REACTION.

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In dealing with nearly neutral solutions of feebly dissociated electrolytes, especially when they are highly coloured, it is frequently convenient to gauge reaction by the use of litmus paper, and so avoid either electric measurement or the introduction of a fluid indicator into a comparatively large volume of fluid. And it has been found that if suitable precautions are taken quite reliable information can be obtained as to the H˙ ion concentration of such fluids in this way. The apparently contradictory results obtained in such cases by using different commercial samples of litmus paper, immersing the paper for a short or a long time in the fluid, using one drop on a piece of paper or a small piece of paper in a considerable volume of fluid, and so on, are capable of simple interpretation.

The precautions to be observed are all those which apply to the use of litmus solution with the addition of those necessitated by the fact that it is applied to paper. As an example of the former class the "neutral salt effect" may be mentioned: the "time factor" is typical of the latter.

EXPERIMENTAL.

The following solutions were used:

Ammonia-ammonium chloride mixtures. A solution of ammonium chloride and ammonia was prepared so as to be approximately 3 N with respect to the former constituent and 3/32 N with respect to the latter. Dilutions with boiled distilled water were made ×2, ×4, ×16, ×32. According to Fels [1904] these solutions should have the same P_H^+ value at 18° C. The values obtained on examination in the hydrogen cell using a calomel electrode were 8·14, 8·14, 8·13, 8·14 respectively, while 8·12 was that obtained for the undiluted material.

Acetic acid-sodium acetate mixtures. A solution of commercial sodium acetate 0·61 N, containing acetic acid corresponding to 0·61 64 N was diluted ×1, ×2, ×8 and ×16. These solutions should have the same P_H^+ value very closely. The values found were 6·25, 6·20, 6·27, 6·23.

Neutral phosphate solution. This was a mixture of 68·8 c.c. of Sörensen's N/15 Na_2HPO_4 solution, and 131·2 c.c. of his N/15 KH_2PO_4 solution. The P_H^+ value determined was 7·05. The figure at present accepted for absolute neutrality is $P_H^+ = 7·07$ [Sörensen, 1909].

Solutions of serum globulin and Witte peptone were also examined, but they presented, in addition to the phenomena observed with solutions free from proteins and their primary disintegration products, others of a more complex and different order. For this reason it was decided to deal with them more fully in a separate communication and cite here only one or two typical cases.

Litmus solution. The reaction between litmus solution and the solution with which it is mixed is practically instantaneous. In saline solutions it gives an indication of the reaction of the solution subject only to a correction for the "neutral salt effect": for it is well known that, for solutions of the same H ion concentration, indicators in the presence of larger quantities of neutral salt give slightly different colours from those which they give when little salt is present [Sörensen, 1912].

The litmus tincture used was itself apparently neutral, or very nearly so, since it gave almost exactly the same tint when added in the same quantity to equal volumes of boiled distilled water and neutral phosphate solution.

The ammonia-ammonium chloride mixtures all gave precisely the same tint as far as could be observed with litmus solution, with the exception of the ×32 dilution, 100 c.c. of which required 0·02 c.c. N/10 NaOH to bring the colour to the same tint as that of the others. The acetic acid-sodium acetate mixtures, on the other hand, gave progressively pinker solutions with litmus tincture as dilution increased. From this observation I think it is legitimate to draw the conclusion that the neutral salt effect is observable in all the acetic acid-acetate mixtures, and only in one case in the ammonia-ammonium chloride mixtures. This is probably due to the fact that the P_H^+ value of the latter mixture is near the alkaline limit of the sensitive range of litmus.

"Equilibrium tint." If a small piece of litmus paper be introduced into a comparatively large volume of any of these solutions it will, after a lapse of a longer or shorter time, assume a definite tint which undergoes no further change. This it is proposed to call the "equilibrium tint" of the paper used in the solution under consideration.

"*Reaction inertia*." A solution whose $H^{\cdot}$ ion concentration is but little affected by the addition of $H^{\cdot}$ or OH' ions, say in the form of hydrochloric acid or caustic soda, can be said to have a greater "reaction inertia" than pure water or a dilute solution of a completely dissociated electrolyte, whose $H^{\cdot}$ ion concentration would be profoundly affected by such an addition.

The above acetic acid-sodium acetate mixtures, although of the same absolute reaction ($P_H^+ = 6\cdot2$), differ very much in reaction inertia, as is in fact patent from their composition. The number of c.c. of N/10 NaOH solution required to bring 100 c.c. of any one solution from $P_H^+ = 6\cdot2$ to $P_H^+ = 8\cdot1$ may be for the purpose of this note considered as a numerical expression of this property. A 50 c.c. sample of each dilution being taken and equal quantities of litmus tincture added to each they presented different colours but had $P_H^+ = 6\cdot2$ in each case as described above. A 50 c.c. sample of an ammonia-ammonium chloride mixture to which an equal quantity of litmus tincture was added furnished a standard tint $P_H^+ = 8\cdot1$. The acetic acid-acetate samples were then titrated with N/10 NaOH to this standard. The results are tabulated:

Acetic acid-sodium acetate mixture.

Dilution	Reaction inertia
1	8
2	4
8	1
16	0·5

In a similar manner titrations of the ammonia-ammonium chloride mixtures were undertaken from $P_H^+ = 8\cdot1$ to $P_H^+ = 6\cdot2$ using N/10 HCl, and from these results a similar table may be prepared. The standard pink tint ($P_H^+ = 6\cdot2$) was that of the undiluted acetic acid-acetate mixture.

Ammonia-ammonium chloride mixture.

Dilution	Reaction inertia
1	90
2	45
4	22
16	6
32	3

With N/15 phosphate mixtures it was found that 20 c.c. of a solution $P_H^+ = 8\cdot1$ required 0·9 c.c. N HCl to bring the reaction to $P_H^+ = 6\cdot2$. The "reaction inertia" for N/15 phosphate solution may then be taken as $0\cdot9 \times 5 \times 10 = 45$.

Observations with Litmus paper.

Four commercial varieties of litmus paper were examined, each providing a "red" and a "blue" paper. Three brands were glazed with the litmus applied to one side only, and one soft and absorbent.

Of the three that were glazed, No. 1 was dyed heavily and apparently loaded with a material of considerable "reaction inertia"; No. 2 was not so heavily dyed though the grain of the paper was similar; No. 3 was faintly dyed on a perfectly smooth paper, presenting a homogeneous appearance. The unglazed paper was labelled No. 4.

Colour tint of paper after 24 hours immersion. Small pieces of each paper about 25 mm.² area were immersed for 24 hours in 240 c.c. of ammonia-ammonium chloride solution of each dilution. At the end of that time all the pieces of paper were of the same tint.

Only one difference could be observed. The heavily loaded paper, No. 1 (blue) was more deeply dyed in the more concentrated solutions than in those more dilute. The adsorption equilibrium seems to shift in the direction of greater adsorption of dye with increasing concentration of salt at constant reaction. A special experiment ratified this conclusion. Pieces of paper No. 1 blue after prolonged immersion in solutions $P_H^+ = 8\cdot1$ containing 15, 7·5, 3·8, 1·9, 0·5 per cent. NaCl gave up progressively more dye to the solution as dilution increased, though in similar solutions of $P_H^+ = 6\cdot1$ no such change could be definitely observed.

Paper No. 1 red immersed in N/15 phosphate solutions $P_H^+ = 4\cdot5, 7\cdot1, 9\cdot2$ respectively gave up very markedly increased quantities of litmus to the solution with increasing alkalinity. In parallel experiments the litmus found in the alkaline solution was 5–6 times that found in the acid solution.

This, in conjunction with many similar observations in this connection, has led me to the conclusion that in similar solutions the adsorption equilibrium changes in the direction of diminished adsorption of litmus by paper in solutions of increasing alkalinity.

In the acetic acid-sodium acetate solutions similarly examined small pieces of each paper gave the same tint after 24 hours immersion, only in one and the same dilution. Passing from one dilution to another it was found that with increasing dilution just that change could be noticed in the equilibrium tint which, as far as could be judged corresponded to the variation in tint with litmus tincture due to the "neutral salt effect" described above.

Observation of rate of change of colour. In the above experiments the

time taken for a piece of litmus paper to acquire its equilibrium tint was seen to vary very considerably. The same paper changed more rapidly in a solution of greater reaction inertia than in one of less reaction inertia in all cases, and, as one would expect, a paper took longer to reach its equilibrium tint when the colour change from its original to its final tint was great. For instance a red paper (No. 1 red) took several hours to come to its equilibrium tint in the most dilute alkaline solution, while a blue paper did so in a few minutes as it was already almost at that tint.

Observation of effect of exposure to the air. It will have been noticed that the two main test mixtures chosen each have a volatile constituent. Pieces of paper that have been immersed for sufficient length of time to assume the equilibrium tint, change that tint rapidly on exposure to the air in such a case. The reddening of blue paper due to the volatilisation of ammonia is naturally more rapid than the blueing of red paper due to acetic acid evaporation.

"Amphoteric solutions." Dealing with approximately neutral solutions a distinction is frequently made between a solution that is " merely neutral " and one that is "amphoteric." Milk, urine, and solutions of phosphates are regarded as typical examples of the latter class and they are said to " turn blue litmus paper red, and red litmus paper blue." What is actually seen may be better described as the " reddening of blue litmus paper and the blueing of red litmus paper." The " merely neutral " solution is one of low reaction inertia. A drop placed on paper wets it but only to a very small extent alters its reaction. The colour of the paper therefore remains unchanged. The " amphoteric" solution, on the other hand, has a high reaction inertia. It usually contains a salt of a feebly dissociated acid or base and, being neutral or nearly so, alters the reaction of litmus paper with which it is brought into contact in the direction of neutrality.

It would naturally be expected that small pieces of paper, red and blue, would, when immersed in a large volume of an amphoteric solution, ultimately assume the same tint. During the first few hours of observation it is seen that the blue paper is becoming progressively redder, and the red paper bluer, but before equilibrium can be attained certain disturbing influences may be encountered. For instance if protein be present the colour of the paper will be interfered with. And, again, since the range of reaction through which the litmus changes is different in the two cases, the losses of litmus to the solution will not be the same. A blue and a red sample of the quickly reacting paper No. 4 were seen to assume very nearly the same tint in a large volume of milk in 24 hours.

Preliminary observations in protein solutions. In solutions of protein, peptone and the like the use of litmus paper has, as far as my experience goes, always indicated the solution to be more alkaline than it really is. For instance an acid 4 per cent. solution of Witte peptone containing 1 per cent. of sodium chloride, and 1 c.c. per cent. of normal hydrochloric acid was found by electric measurement to have $P_H^+ = 6.5$. In a tintometer arranged to compensate for the colour of the Witte peptone [Walpole, 1910] and using neutral red as indicator, the P_H^+ value was made out to be the same as that of a phosphate mixture $P = 6.8$[1]. A neutral $N/15$ phosphate solution was prepared ($P_H^+ = 7.07$) and a comparison instituted between this solution and the acid Witte peptone solution above. It will have been noticed that neither contains a volatile constituent.

Three papers were taken (No. 4 red, No. 2 blue and No. 3 red) and one slip of each paper dropped into a large volume of each fluid. They were removed at intervals, laid on white paper and examined in diffused daylight.

The unglazed, quickly-reacting paper (No. 4 red) gave with the acid peptone solution a bluer tinge almost at once than the corresponding paper in the phosphate solution and this relation continued indefinitely. The blue paper No. 2 was always bluer in the acid peptone solution: the red paper No. 3 lost most of its colour in the phosphate solution before the progressively bluer paper in the acid Witte peptone solution could match it.

In another case a faintly alkaline 4 per cent. solution of Witte peptone of reaction $P_H^+ = 7.3$ gave with papers No. 2 (red and blue), No. 3 (red and blue), No. 4 (red and blue) the same equilibrium tint, as nearly as could be judged, as any of the ammonia-ammonium chloride dilutions examined, which had $P_H^+ = 8.1$ in each case.

EXAMPLES.

By neglecting to consider these secondary influences it is possible to obtain some bewildering results. For instance we may take two alkaline solutions having the same reaction, $P_H^+ = 8.1$; A is a solution of ammonium chloride and ammonia, $3N$ with respect to the former and $3/32$ N with respect to the latter; B is this solution diluted 32 times and the "reaction inertia" of A is about 30 times that of B; C is a neutral phosphate solution;

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[1] In the same apparatus using litmus tincture as indicator no real match could be obtained but the indications were that the peptone solution was more alkaline than a phosphate mixture $P_H^+ = 9$.

and D is pure water. Red litmus paper No. 1 dipped into the solutions, removed, and examined shows at once a bright blue colour with A, red with B, a bluish tinge with C and red with D. Blue paper No. 4 shows blue rapidly turning red with A, blue slowly turning not quite so red with B, red with C, and blue with D; while blue paper No. 2 shows with A and B blue very slowly turning red, and with C and D slight change towards red, less marked in the latter case.

The following interpretations suggest themselves. With red paper No. 1 the concentrated solution A neutralises the acid red material of the heavily loaded paper, and in spite of the glazing shows a pronounced blue colour at once; with B the reaction of the absorbed fluid is determined more by the paper than by the substances originally dissolved in the water, and the evaporation of the free ammonia has been almost complete long before any great effect would have been observed even if the paper had been bathed in an excess of solution. Solution C gives the neutral tint appearing blue on a red paper, while distilled water leaves the paper unaffected.

With blue paper No. 4 the attainment of equilibrium is very much more rapid because the paper is not glazed, and the rate of evaporation of ammonia is also accelerated though to nothing like the same extent. Blue paper No. 2 has a smaller "reaction inertia" than No. 1, besides it is already blue. It differs from No. 4 in that it is glazed. The instances cited above are exaggerated and, in the main, their interpretation obvious; but these factors operating in a minor degree may lead to incorrect conclusions.

SUMMARY.

Except only in so far as the colour is influenced by the presence of neutral salts, the reaction of a solution is indicated by the colour of a piece of litmus paper which has been immersed in an excess of the fluid until no further change can be observed. The correction for neutral salt effect is a small one and is of the same order as that applying to litmus solution when used in the same way.

When the *quantity of solution used is limited in amount*, as when a drop of solution is placed on the paper and the effect observed, the following secondary influences determine the colour changes seen—

(1) Gradual attainment of reaction equilibrium between the litmus on the paper and the solution; more rapid with unglazed paper than with paper that is glazed.

(2) The actual effect of the reaction of the material in the paper on the

limited amount of solution used. This will, with the same paper, be most marked with a solution of lowest "reaction inertia." It is obviously fallacious to test the reaction of tap water by watching the effect of one drop of water on a heavily dyed paper.

(3) The "reaction inertia" of the paper. A thick heavily loaded paper has more effect in modifying the reaction of a drop or two of a solution than a thin paper lightly dyed with a litmus solution of low reaction inertia.

(4) The effect of exposure to the air. The evaporation or oxidation of some constituent of the solution affecting its reaction, will naturally produce a corresponding effect.

(5) The liberation of indicator from the paper, which is greater in the absence of neutral electrolytes and is more marked in alkaline than in acid solutions.

Determination of reaction of nearly neutral solutions of *proteins* and *their decomposition products* by means of litmus paper is very difficult. Such cases have not been dealt with fully here, but will form the subject of a later communication. The general effect is to indicate that the solution is more alkaline than it really is.

The same phenomena may be observed with litmus tincture to a much more marked degree. For this reason it has been frequently described as useless in such cases.

REFERENCES.

Fels (1904), *Zeitsch. Electrochem.* **10**, 208.
Sörensen (1909), *Biochem. Zeitsch.* **21**, 131.
—— (1912), *Ergebnisse Physiol.* **12**, 441.
Walpole (1910), *J. Physiol.* **40**, 27.

XXVII. THE PREPARATION FROM ANIMAL TISSUES OF A SUBSTANCE WHICH CURES POLYNEURITIS IN BIRDS INDUCED BY DIETS OF POLISHED RICE.

PART I.

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Although several workers have isolated from various food-stuffs substances capable in small amount of curing polyneuritis in pigeons and have ascribed chemical formulae to them, attempts to prepare the active constituent in an amount sufficient for investigating its chemical constitution and properties have up to the present time been unsuccessful.

Funk [1911, 1913] has isolated from rice polishings, yeast, milk, and ox-brain substances curative in oral doses of 0·02 g. and possessing similar melting-points.

Edie, Evans, Moore, Simpson, and Webster [1912] have also prepared a substance from yeast curative in small amount, to which they have ascribed the formula $C_7H_{17}O_5N_2$, and Suzuki, Shimamura, and Odaki [1912] isolated an active substance containing nitrogen from rice-polishings in the form of a picrate, but they have not published any results of its analysis.

1. ISOLATION OF AN ANTI-NEURITIC SUBSTANCE FROM HORSE-FLESH.

In the present communication a method is described for preparing from horse-flesh a substance capable in minute amount of curing polyneuritis in pigeons.

The method employed was a modification of that described by Maclean [1912, 1] for the isolation and purification of the phosphatides of the kidney. The horse-flesh was minced, dried at 30° by means of an electric fan and ground, and the dry powder (weight 4000 g.) extracted at 37° with absolute alcohol on a shaking machine. The filtered extract was evaporated at 40° *in*

vacuo to remove the alcohol. The extract (weight 500 g.) was found to possess marked curative properties towards pigeons affected with polyneuritis, 4 g. being sufficient to bring about complete recovery within 24 hours. The extract was then treated with an excess of ether and the mixture allowed to stand in the cold room for 12 hours. The ether dissolved the fats and lipoids. but left undissolved a considerable amount of a white substance. This was washed thoroughly with ether and then tested on neuritic pigeons. Doses of 0·3 g. were sufficient to bring about complete recovery within 12 hours. This procedure therefore afforded a simple technique for separating a highly curative fraction from fats and lipoids and it was much more convenient than extracting the active substance by means of water.

The ether-soluble fraction was freed from ether by evaporating *in vacuo*. As much as 12 g. of the residue possessed only slight curative properties, while 2 g. of the lipoids separated from this fraction by means of acetone were inactive, but 7 g. were curative within 24 hours. It was thus possible to separate the bulk of the anti-neuritic substance from the alcoholic extract of horse-flesh by means of ether. These results support the explanation advanced by Maclean [1912, 2] for the curative properties of lecithin observed by several workers, namely, that the anti-neuritic substance is not a lipoid, but is present in ordinary lecithin as an impurity readily extractable therefrom by simple methods.

The ether-insoluble fraction was next treated with absolute alcohol, by which a large portion was dissolved. The insoluble residue contained both inorganic and organic material, but possessed no curative properties. The alcohol-soluble fraction however was strongly curative, and it was treated with excess of ether. This caused the separation of a yellow syrup (yield 50 g.) which was completely soluble in water and 0·2 g. of which was sufficient to cure pigeons affected with polyneuritis. The aqueous solution of the syrup was allowed to stand in a vacuum desiccator and a white crystalline substance gradually separated. This proved to be carnine and possessed no curative properties. The filtrate, which was highly curative, was next treated with finely powdered lead acetate, until there was no more separation of a flocculent precipitate, and the mixture was allowed to stand for 12 hours. The precipitate was filtered off, washed with water at 35°, decomposed with sulphuric acid, and the excess of acid removed by the careful addition of baryta. The filtered solution possessed no curative properties. The filtrate from the lead acetate precipitation was freed from lead by careful treatment with dilute sulphuric acid and was left very slightly acid. It possessed curative properties and was next treated with silver nitrate, which produced

a copious yellowish-white precipitate. This was filtered off, decomposed by hydrochloric acid and the resulting solution was nearly neutralised with soda and was found to be curative. The filtrate from the silver nitrate precipitation was also curative, but its content of active substance was completely precipitated by silver nitrate when baryta was added. By carrying out several animal experiments it was found that at least 3/5 of the total amount of anti-neuritic substance present in the filtrate from the lead acetate precipitation was precipitated by the addition of silver nitrate only, and about 1/4 was carried out of solution by the subsequent addition of baryta. The remaining 3/20 was probably destroyed by the alkali. The residue obtained by the evaporation of the curative solution resulting from the decomposition of the first silver nitrate precipitate with hydrochloric acid was next extracted with chloroform. Only a small amount of substance was extracted and this possessed no curative properties. The anti-neuritic substance was therefore insoluble in chloroform. It was also found to be insoluble in benzene and ethyl acetate. The residue insoluble in chloroform was next extracted with absolute alcohol; a large amount was insoluble, but the curative substance was found to have been entirely dissolved, and 0·10 g. of the soluble fraction was sufficient to cure a pigeon affected with polyneuritis. This fraction was then extracted with acetone and a considerable amount was dissolved. The insoluble residue, which contained all the active substance, was dissolved in 50 % alcohol, and the solution allowed to stand in a desiccator for some days. A white substance gradually crystallised out, 0·06 g. of which administered to each of two pigeons ameliorated the symptoms of polyneuritis in a few hours and effected a complete recovery within 24 hours. The substance dissolved with moderate readiness in water, but was insoluble in absolute alcohol.

It was not possible to obtain a sufficient amount of the active substance to determine its chemical composition, and the work is therefore being repeated on a much larger scale. As, however, it was found [Cooper, 1913] that cardiac muscle contained considerably more anti-neuritic substance than voluntary muscle, ox-heart has been substituted for the horse-flesh and the results so far obtained justify this change.

Some experiments have also been carried out to ascertain if the anti-neuritic substance could be precipitated by means of ether from the fats and lipoids derived from other animal-tissues. It was found that considerable amounts of curative fractions could be obtained by this procedure not only from horse-flesh and ox-heart, but also from horse-kidney, beef, ox-brain and liver.

Only a small amount of material, however, was separated by ether from the alcoholic extract of egg-yolk, and its curative power was very feeble. The results indicate that this fractionation affords quite a general method for separating the anti-neuritic substance from the fats and lipoids of animal tissues.

2. The Absorptive Capacity of Animal Charcoal for the Anti-neuritic Substance.

Chamberlain and Vedder [1911, 1912] found that an extract of rice-polishings containing the anti-neuritic substance after being filtered through bone-black could no longer prevent polyneuritis in fowls and that the active substance could only be extracted to a small extent from the charcoal by water or alcohol. As these results are of great importance, the experiments have been repeated. Powdered animal charcoal was extracted three times with boiling water and then dried. A curative solution of known activity (prepared by dissolving the ether-insoluble fraction derived from an alcoholic extract of horse-flesh in water) was filtered slowly through a bed of the charcoal six times. The filtrate was still curative, but its activity was reduced to the extent of 30 %. Separate portions of the charcoal were then extracted with water and alcohol, and the extracts were found to be highly curative. The anti-neuritic substance was thus partially absorbed by charcoal and could be recovered from the latter by extraction with water or alcohol. It is seen that the results do not agree entirely with those obtained by Chamberlain and Vedder, but it is unlikely that the use of bone-black as an absorptive agent will be of much value for the isolation of the anti-neuritic substance.

3. The Effect of Sulphuretted Hydrogen upon the Active Substance.

In a preliminary fractionation of the horse-flesh extract sulphuretted hydrogen was employed to decompose the lead and silver precipitates and to remove the excess of the metals from solution, and during these processes a rapid disappearance of the active substance occurred. By substituting acids for the sulphuretted hydrogen (sulphuric acid for lead, hydrochloric acid for silver) it was possible to conduct the operations with a much smaller loss of active material.

These facts suggested that sulphuretted hydrogen destroyed the anti-neuritic substance. Accordingly, the effect upon activity of passing this gas

through a curative solution for four hours was investigated, but no evidence was obtained of any destruction of the anti-neuritic substance. The diminished activity during the above manipulations would therefore appear to have been due to the absorption of the curative substance by the colloidal metallic sulphides.

4. The Effect of Alkali upon the Active Substance.

Several investigators have observed that a considerable loss of anti-neuritic substance occurs when chemical operations involving the use of alkali are employed in the fractionations. It was thought desirable to throw some light on this matter by quantitatively studying the effect of treatment with alkali upon the curative power of the substance.

To a solution of known activity ammonia was added until its concentration throughout the total volume of liquid reached 10 %. The mixture was kept in a closed flask at ordinary temperatures for 24 hours, the ammonia then removed by a current of air, and the curative power of the solution redetermined. It was found that 50 % of the anti-neuritic substance had become destroyed by contact with the alkali.

These results indicate that the isolation of the anti-neuritic substance in large amount is only likely to be effected by employing chemical methods that do not involve the use of alkali and sulphuretted hydrogen.

5. The Effect of Alkaloids upon Pigeons affected with Polyneuritis.

Strychnine was found [Cooper, 1913] to prolong the lives of birds affected with polyneuritis but to exert no curative action. It was accordingly of interest to investigate the action of other alkaloids. Experiments have been carried out with quinine, cinchonine, and cinchonidine. The substances were dissolved in a trace of hydrochloric acid and administered to the birds orally.

Doses of quinine ranging from 0·01 to 0·1 g. exerted a temporary curative action. In six cases the symptoms were ameliorated for about three days and then became once more acute, further administration of quinine having no effect. In two other cases however the neuritic symptoms completely disappeared within a few hours, and one of the birds remained free from polyneuritis for four days, whilst the other, receiving daily in addition to polished rice 0·025 g. of quinine, remained healthy for 10 days and then died exhibiting

symptoms merely of weakness and not of acute polyneuritis. When given in amounts exceeding 0·1 g. however quinine had no ameliorative effect, but actually appeared to hasten the fatal issue.

Daily doses of quinine ranging from 0·01 to 0·025 g. administered to several pigeons fed on polished rice did not prevent or delay the development of neuritic systems.

Cinchonine in doses varying from 0·05 to 0·1 g. like quinine effected a marked improvement in the condition of pigeons suffering from polyneuritis, but within 48 hours the birds again exhibited the acute neuritic symptoms and died notwithstanding further doses of the alkaloid. Cinchonidine on the other hand had no ameliorative effect at all.

As quinine and cinchonine are extracted from cinchona-bark by means of acid, it was thought that their curative action might possibly be due to contamination with traces of the anti-neuritic substance present in the plants. The active substance is readily destroyed by heat, so that some light could be thrown on this matter by observing whether the alkaloids retained their curative properties after strong heating. It was actually found that after being heated at 125° for six hours quinine no longer had any ameliorative effect upon neuritic pigeons. As the alkaloid is not chemically altered by such treatment, it would appear that its curative action is due to the presence as an impurity of a minute amount of the anti-neuritic substance.

6. The Effect of Alcohol upon Birds fed on Polished Rice.

The administration of small doses of alcohol (0·5 c.c. three times daily) to pigeons fed on polished rice had no measurable effect upon the period of time elapsing before the occurrence of symptoms of polyneuritis, and thus appeared not to influence the utilisation of the supply of anti-neuritic substance distributed in the tissues of the birds.

This is presumptive evidence that alcoholic neuritis is not caused by any diminished capacity on the part of the organism to utilise the anti-neuritic substance which might be expected to result from the disturbing effects of alcohol upon the metabolism of the nervous system.

Summary.

1. A fraction rich in the anti-neuritic substance can be precipitated from the fats and lipoids (alcoholic extracts) of various animal tissues by means of ether.

2. A method based on this observation is described for isolating from horse-flesh a substance small amounts of which can cure polyneuritis in pigeons.

3. The substance is insoluble in absolute alcohol, benzene, chloroform, ether, and ethyl acetate, but is moderately soluble in water.

4. The substance is absorbed to some extent by animal charcoal and is readily destroyed by alkali. It is not inactivated by sulphuretted hydrogen, but disappears in large amounts during chemical operations in which colloidal metallic sulphides are formed.

5. Quinine and cinchonine exert a temporary curative action upon birds affected with polyneuritis. After being heated at 125° for 6 hours, however, quinine has no ameliorative effect, so that its curative properties would appear to be due to its contamination with traces of the anti-neuritic substance derived from the cinchona bark.

6. The administration of small doses of alcohol to birds fed on polished rice does not affect the period of time elapsing before the occurrence of poly-neuritis, and thus appears not to influence the utilisation of the supply of anti-neuritic substance distributed in the tissues of the birds. This suggests that alcoholic neuritis does not result from a diminished capacity of the organism to utilise the anti-neuritic substance.

In conclusion I am glad to have this opportunity of expressing my indebtedness to Dr Hugh MacLean for drawing attention to the curative properties of the residues obtained by treating alcoholic extracts of voluntary muscle with ether and for much valuable help in the course of the investigation.

REFERENCES.

Chamberlain and Vedder (1911), *Philipp. J. Science*, **6**, 395.
—— —— (1912), *Phillip. J. Science*, **7**, 39.
Cooper (1913), *J. Hyg.* **12**, 436.
Edie, Evans, Moore, Simpson, and Webster (1912), *Biochem. J.* **6**, 234.
Funk (1911), *J. Physiol.* **43**, 395.
—— (1913), *J. Physiol.* **45**, 75.
MacLean (1912, 1), *Biochem. J.* **6**, 333.
—— (1912, 2), *Biochem. J.* **6**, 355.
Suzuki, Shimamura and Odaki (1912), *Biochem. Zeitsch.* **43**, 89.

XXVIII. ON THE LIPOLYTIC ACTION OF THE BLOOD.

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Our knowledge of the ultimate processes involved in the storage of fat and its mode of transport from the fat depots to the tissues requiring it is very scanty. The fate of fat from the intestine to its presence in the thoracic duct is accurately known from the experiments of Moore [1897]. From the thoracic duct it passes into the blood and is rapidly converted into a substance no longer extractable by ether, which can pass through cell membranes to be stored up as fat in the fat depots or to be used as a source of energy. Of the nature of this substance our knowledge is practically nil. It has been shown by numerous observers that the tissues contain a " fat-splitting " ferment, the action of which under certain circumstances may also be reversible, and it was considered probable that the blood serum contained a similar ferment. This view was first of all advanced by Hanriot [1896], who tried the action of the blood on monobutyrin. His experiments, however, are erroneous as has been pointed out by Arthus [1902] because he took no precautions to ensure aseptic conditions and also because he used monobutyrin, which can be very easily hydrolysed, and it by no means follows that ferments, breaking up such simple esters as monobutyrin, have the same effect on neutral fats. Hagemeister [1903] showed that the injection of fat between muscle fibres was followed by its presence beneath the sarcolemma. He deduced that the lipolytic ferment of the tissue fluids caused the hydrolysis of the fat, diffusion of the resulting products occurring, followed by resynthesis in the muscle.

Fischler [1902, 1903] demonstrated the deposition of "fat" in organs after the injection of soap into the blood, or the perfusion of surviving organs with blood containing soap, thus lending support to the above. Munk [quoted by Fischler, 1902, p. 149], however, has shown that soap

even in small amounts exerts a toxic effect on the medullary centres. A small amount of soap apparently does occur in the blood and according to Schultz [1897] 28 % of the total fatty acids in the blood are in the form of soaps.

There is therefore no *a priori* objection to the possibility of the blood containing a lipase acting on the fats in the above-mentioned way and of the products being rapidly diffused into the cells to be resynthesised and stored up or used as required.

Experiments, however, conducted with strict precautions as to asepsis show that the blood serum does not contain a ferment capable of splitting ordinary fats.

The experiments of Cohnstein and Michaelis [1894] showed that there was a loss of ether-soluble substances on incubating mixtures of blood and chyle and a corresponding increase in water-soluble substances, and that this was due to a ferment in the erythrocytes. Their experiments have, however, the drawbacks that they were not absolutely aseptic and that there were inaccuracies in their mode of estimation.

Their results have been in part confirmed by Hamburger [1900] who was able to demonstrate a loss of ether-soluble substance after incubating a lipanin emulsion with blood.

Weigert [1900], however, considered that Cohnstein and Michaelis' results could be explained by autolysis of the lecithin. Hepner [1898] had previously shown that the blood plasma contains cholesterol and cholesterol esters of the fatty acids, and thought that the free cholesterol might conceivably have arisen by the action of a ferment on the cholesterol ester. Weigert therefore thought that the loss in ether extractives in Cohnstein and Michaelis' experiments could be accounted for by this, since the liberated fatty acids would become converted into soaps and thus no longer be ether-soluble. He experimented with blood plasma, blood and corpuscles alone. He found that blood plasma on incubation suffered no loss; when blood was incubated a loss of 18 % of ether extract occurred; whereas when corpuscles alone were thus treated there was a loss of 40–50 % of ether extract, and since Hepner and Hoppe-Seyler [quoted by Hepner, 1898] showed that red cells contained no cholesterol ester or neutral fat, the resulting change can only have been due to hydrolysis of the lecithin.

In the experiments of the author the following methods of extracting fat were adopted:

1. Drying the material with sand over a water-bath, and extracting with ether in a Soxhlet apparatus for 48 hours.

2. Mixing with anhydrous sodium sulphate as recommended by Pinkus, finely powdering the resulting dry mass, and extracting with ether at the room temperature for several days, with vigorous shaking at intervals. The extraction was repeated with fresh ether till nothing further was extracted.

3. Mixing with 94 % alcohol and keeping at 37° for several days, then filtering and repeatedly extracting the precipitate with alcohol, and finally boiling up. The united extracts were then evaporated and extracted with 94 % alcohol. The original residue was also extracted with ether, and the resulting extracts were divided into their constituents: fats, fatty acids and lecithin.

4. Digesting with pepsin and hydrochloric acid; evaporating with sand and extracting in the Soxhlet with ether.

Precautions to preserve absolute asepsis were taken in all these experiments. The various gases were allowed to bubble through the mixtures after having been filtered through sterile cotton wool. All specimens showing bacterial contamination, either by direct or cultural examination, were discarded.

I. Changes occurring when Blood alone is incubated.

Exp. 1.

100 c.c. of blood (by sand and ether extraction) yielded			...	...	0·403 g. fat
100 ,, ,, incubated at 37° for 24 hours in closed vessel yielded			...		0·381 ,,
100 ,, ,, same incubation, but with air current			...	...	0·297 ,,

Loss in specimen in air current = 26·3 %.
,, without ,, = 5·4 %.

Exp. 2.

100 c.c. of blood by sand and ether extraction yielded		·	...	...	0·366 g. fat.
100 ,, ,, incubated in air current for 24 hours at 37° ...				...	0·295 ,,
100 ,, ,, incubated without air at 37° for 24 hours			...	...	0·299 ,,

Loss in air current = 19·4 %.
,, without ,, = 18·3 %.

Exp. 3. Extraction after drying with anhydrous sodium sulphate.

(a) 100 c.c. of blood contained ... ... 0·24 g. fat.
100 ,, ,, after incubation in air current for 24 hours at 37° contained ... ... 0·184 ,,

Loss = 23·3 %.

(b) 100 c.c. of blood contained ... ... 0·328 g. fat.
100 ,, ,, treated as in (a) contained ... ... 0·276 ,,

Loss = 15·9 %.

For the purpose of determining to what the loss was due, the following estimations were made. The blood was extracted with alcohol and the residue with ether, the alcoholic extract was acidulated with dilute mineral

acid evaporated and shaken out with ether. The ether was freed from mineral acid by repeatedly shaking with water. The ethereal residues were united, evaporated, dried and weighed. The lecithin was determined by precipitation with acetone, the fatty acids by titration with N/10 alcoholic potash, and the neutral fat subsequently by boiling with a known quantity of N/10 absolute alcoholic potash.

250 c.c. of blood were taken.

	Unincubated blood		Incubated blood	
Total weight of residue	0·958 g.		0·936 g.	
Lecithin	0·28		0·127	
Fatty acids	0·152	(5·4 c.c. N/10 alc. pot.)	0·245	(8·7 c.c.)
Neutral fat	0·316	(11·2 ,, ,,)	0·310	(11·0 c.c.)
Cholesterol	0·102		0·122	

With the exception of the increase in the cholesterol figure the whole difference is in the lecithin and fatty acids—the loss in the lecithin is 0·153 g., and the increase in the fatty acids is 0·093 g. Now if lecithin contains about 60 % fatty acids, then 0·153 g. contains 0·092 fatty acid.

Several other experiments showed a similar change.

II. Chyle Alone.

Exp. 1.

50 c.c. of chyle contained (by sand and ether method)			...	0·631 g. of fat.	
50 ,, ,, incubated at 37° without current of air			...	0·579 ,,	
100 ,, ,, incubated at 37° in air current		...	...	1·064 ,,	

Loss without air current = 8·2 %.
,,   in   ,,   ,,   = 15·7 %.

Exp. 2. *Extracted by the Sodium Sulphate-Ether method.*

25 c.c. of chyle contained	...	...	...	...	0·317 g. of fat.
100 ,, ,, incubated in air current contained ...			...	...	1·178 ,,

Loss = 7·1 %.

Exp. 3. *Alcohol-ether extraction.*

	(a) Unincubated	(b) Incubated
100 c.c. of chyle		
Ethereal extract of the original extract	1·356 g.	1·243 g. (loss = 8·4 %)

After acidulating the residue of the original extract, shaking out with ether and removing mineral acid and adding the ethereal extracts together;

Lecithin found	0·056 g.	0·014 g.
Fatty acids	0·0	0·034
Neutral fat	1·208	1·137

From this it would appear that there was some hydrolysis of the lecithin and loss of a small amount of neutral fat. The fatty acid increase can be explained by the hydrolysis of the lecithin, since 0·048 g. lecithin contains about 0·029 g. of fatty acid.

III. Experiments on the Incubation of a Mixture of Chyle and Blood.

(1) The sand method of extraction.

Exp. 1.

100 c.c. of a mixture of equal parts of chyle and blood were found to contain   1·281 g. of fat.
100 c.c. of same mixture incubated for 24 hours without air current were found
to contain   ...   .   1·200   ,,
100 c.c. of same, incubated in an air current for 24 hours contained   ...   0·9155 ,,
100 c.c. of same, incubated in an air current for 48 hours contained   ...   0·8025 ,,

Loss was therefore:—in incubation without air current   6·4 %,
in 24 hours incubation with air current 28·5 %,
in 48   ,,   ,,   ,,   ,,   37·3 %.

Exp. 2.   Performed as above, showed;

100 c.c. of a blood chyle mixture contained   ...   .   1·41 g. of fat.
100 c.c. of same incubated in air current at 37° for 24 hours   ...   ...   0·536   ,,
100 c.c. of same, incubated for 48 hours, same way   ...   .   0·373   ,,

Loss after 24 hours was 61·9 %.
,,   48   ,,   73·5 %.

(2) Sodium Sulphate Method.

100 c.c. of a mixture of blood and chyle contained   ...   .   1·228 g. of fat.
100 c.c. of the same mixture, incubated in a current of air at 48° for 24 hours   0·661   ,,

Loss = 46·2 %.

These figures show that on incubating chyle and blood together there is a large loss of ether extractive. This is not due to the method of extraction, it is as apparent with the one method as with the other. The loss is greater after air has been passed through the mixture, but contrary to the observation of Cohnstein and Michaelis [1894] it also occurs in the absence of an air current. The loss again is not the sum of the losses that would be obtained by incubating the blood and chyle separately.

Thus in the following experiments:

100 c.c. of blood yielded   ...   .   0·29 g. of fat.
100 c.c. incubated for 24 hours with current of air at 37° yielded ...   ...   0·232   ,,

Loss = 0·058 g. or 20 %.

100 c.c. of chyle yielded   ...   .   1·202   ,,
100 c.c. incubated as above   ...   .   1·034   ,,

Loss = 0·168 g. = 14·0 %.

Now a mixture of 50 c.c. of each contained   ...   .   0·770 g. of fat.
But incubation as before yielded   ...   .   0·478   ,,

Loss = 0·298 g. = 38·4 %.

Now the sum of the individual losses for 50 c.c. of each = 0·117 g., which is less than half the loss which occurred, when the two were incubated together.

This is also shown by the following experiment:

100 c.c. of chyle were found to contain	...	...	...	...	1·268 g. of fat.
100 ,, ,, yielded after incubation	...	...	...	...	1·168 ,,

<div align="center">Loss = 0·1 g.</div>

100 c.c. of blood yielded	...	...	...	...	0·314 ,,
100 ,, ,, after incubation yielded	...	...	...	...	0·244 ,,

<div align="center">Loss = 0·070 g.</div>

A mixture of 50 c.c. of chyle and 25 c.c. of blood were estimated to contain	...	0·712 ,,
After incubation a similar amount yielded ...	...	0·329 ,,

<div align="center">Loss = 0·38 g. = 53·8 %,</div>

whereas the sum of the losses for each separate incubation

$$= 0·05 + 0·0175 = 0·0675 \text{ g.}$$

The loss is thus considerably greater than the sum of the individual losses.

(3)   To see if the serum contained the substance which caused this loss of substance extractable by ether a mixture was made of 100 c.c. of chyle and 100 c.c. of sheep's serum.

100 c.c. of the mixture contained	...	...	...	0·663 g. of fat.
100 ,, ,, ,, after incubation in an air stream after 24 hours yielded				0·591 ,,

<div align="center">Loss = 0·072 g. = 10·8 %.</div>

Similar experiments give like results up to 15 %.

(4)   In order to determine whether the change is due to a ferment in the red corpuscles or other formed constituents, the following experiments were performed with carefully washed erythrocytes. These were obtained by centrifuging whipped blood and washing six times with sterile normal saline in sterile centrifuge tubes.

Exp. 1.

50 c.c. of chyle and 5 c.c. of red cells contained	...	...	...	0·802 g. of fat.
100 ,, ,, 10 ,, ,, incubated for 24 hours in an air current				
yielded	...	...	...	1·172 ,,

<div align="center">Loss = 0·43 g. = 26·9 %.</div>

In the next part of the experiment the chyle was first heated to 80° for 15 minutes:

100 c.c. of this with 10 c.c. of the suspension of red cells incubated for 24 hours				
at 37° yielded	...	...	...	1·107 g. of fat.

<div align="center">Loss for unboiled chyle was 26·9 %.</div>
<div align="center">,, boiled ,, 30·9 %.</div>

Exp. 2.

100 c.c. of chyle yielded	...	...	...	...	1·825 g. of fat.
20 c.c. of red corpuscles yielded		...	...	...	0·048 ,,

100 c.c. of chyle + 20 c.c. of the corpuscles incubated for 24 hours in an air
current yielded   ...   .   1·284 ,,

Loss was 31·4 %.

Exp. 3.

50 c.c. of chyle yielded	...	...	...	...	0·912 g. of fat.
20 c.c. of corpuscle suspension yielded	...	...	...	...	0·048 ,,

Both were heated to 80° and then incubated at 37° for 24 hours. The total amount of ether extract found was 0·937 g. The loss was therefore practically nil.

It will thus be seen that probably the red cells, or substances centrifugalised down with them, have the power of causing this loss of ether extractive.

(5) The next question investigated was whether this power was due to the haemoglobin, to a ferment in the cells, which may be to some extent diffusible into the plasma, or as stated above to elements precipitated by the centrifuge with the red corpuscles.

This was investigated by obtaining crystals of haemoglobin from dog's blood. The blood was whipped and centrifugalised, the corpuscles were then repeatedly washed with normal saline and then laked with ether, centrifugalised and washed with dilute alcohol and ammonium sulphate solution.

Exp. 1.

50 c.c. of chyle + 25 c.c. of a watery Hb solution yielded   ...   ...   0·550 ether ext.
Twice this amount was incubated at 48° for 24 hours in air current and
yielded   ...   .   0·729   ,,

Loss was 0·371 g.

Exp. 2. The haemoglobin was previously treated with a saturated solution of sodium fluoride in a saturated ammonium sulphate solution. The crystals were then repeatedly washed with a saturated solution of ammonium sulphate solution to remove the sodium fluoride.

50 c.c. chyle together with 50 c.c. watery Hb solution yielded   ...   ...   0·665 g. of fat.
The same amount after 24 hours incubation in a stream of air at 48° yielded...   0·632   ,,

Loss was 0·033 g. = 5 %.

Exp. 3.

100 c.c. of chyle was mixed with 50 c.c. of a watery solution of Hb. The Hb
had been previously dried for several days at 50°. The mixture contained   1·268 g. of fat.
The same amount was incubated for 24 hours and yielded   ...   ...   1·202   ,,

Loss = 0·056 g. = 5·2 %.

To elucidate this point, whether the loss was due to the action of the haemoglobin, a mixture of chyle and blood was incubated and a mixed stream of carbonic oxide and oxygen gases was passed through the fluid. The carbonic oxide converted the haemoglobin into carboxy-haemoglobin and the oxygen was available if necessary.

By previous experimentation it was determined that the "lipase" obtained from pig's liver was unaffected by prolonged exposure to a stream of carbonic oxide gas as regards its action on ethyl butyrate.

The blood used was first saturated with carbonic oxide.

Exp. 1.
50 c.c. of blood and chyle yielded ... ... 0·490 g. of ether ext.
50 c.c. of the same mixture after incubation yielded ... ... 0·390 ,,   ,,
Loss was 0·1 g. = 20·4 %.

Exp. 2.
100 c.c. of a mixture of equal parts of blood and chyle yielded ... 1·096 g. of ether ext.
The same amount after similar treatment yielded ... ... 0·731 ,,   ,, .
Loss was 0·365 g. = 33·3 %.

The experiments show that the change can still occur when the haemoglobin is put out of action as an oxygen carrier.

In these experiments it was certain that the haemoglobin was converted into carboxy-haemoglobin because of the colour of the mixture.

In the method of preparation of the haemoglobin crystals, proteins in the corpuscles or substances in the plasma like blood, dust particles, and leucocyte debris may be precipitated so that any possible ferment in connection with them would be precipitated as well. The action of the carbonic oxide and sodium fluoride leads to the supposition that there is a ferment separate from the haemoglobin.

(6) The following experiments were performed to see if the oxygen is really a necessity, or if the air current acts simply mechanically, thoroughly mixing the blood and chyle. The mixtures were incubated for 24 hours at 37°.

(a) 50 c.c. of a mixture of blood and chyle yielded ... ... 0·350 g. of fat.
100 c.c. of this mixture after treatment in coal gas yielded ... ... 0·542 ,,
Loss was therefore 0·158 g. = 22·6 %.

(b) 50 c.c. of blood and chyle contained ... ... 0·450 ,,
100 c.c. incubated in a stream of hydrogen gas contained ... ... 0·696 ,,
Loss was 0·204 g. = 22·5 %.

(c) 50 c.c. of blood and chyle mixture contained ... ... 0·395 ,,
100 c.c. of the same mixture in a hydrogen stream yielded ... ... 0·608 ,,
Loss was 0·182 g. = 23 %.

(d) 50 c.c. of chyle + 25 c.c. of blood contained ... ... 0·673 ,,
The same mixture incubated in air stream yielded ... ... 0·229 ,,
The same mixture incubated in CO_2 stream yielded ... ... 0·478 ,,
Loss in air = 0·444 = 66 %.
,, CO_2 = 0·195 = 29 %.

Thus it would appear that oxygen aids the action of the ferment, but on comparing with the result in (c) of this series it is obvious that mechanical agitation by the passage of a current of any gas also appreciably aids the process.

(7) The influence of protoplasmic and ferment poisons was next examined.

Exp. 1.

(a) 100 c.c. of a mixture of chyle and blood yielded ... ... 0·708 g. of fat.
100 c.c. of same mixture containing 5 % sodium fluoride was incubated
for 24 hours in air stream at 48° and then contained ... ... 0·657 ,,
Loss was 0·051 g. = 7 %.

(b) 100 c.c. of the same mixture with potassium cyanide added and incubated
in the same way yielded ... ... 0·641 ,,
Loss was 0·067 = 10 %.

(c) 200 c.c. of the same mixture was incubated in the ordinary way and yielded 0·547 ,,
Loss was 0·869 = 62 %.

(8) Some experiments were performed to determine the fate of the individual constituents.

Exp. 1. A mixture of equal parts of blood and chyle was made. (a) 100 c.c. were directly dried with sand and extracted with ether in the Soxhlet; (b) 100 c.c. were similarly treated after incubation in an air current at 37° for 24 hours.

(a) Unincubated		(b) Incubated	
Total residue	0·732 g.	0·416 g.	
Neutral fats	0·583 (19·8 c.c.)	0·327 (11·1 c.c. N/10 alk.)	
Fatty acid	0·0	0·0	
Lecithin	0·096	0·032	
Cholesterol	0·053	0·057	

There is thus, as was expected, a loss in neutral fat. The dried residue was then extracted with alcohol in the incubator at 37° while being continuously shaken, the alcoholic extract was evaporated and treated with mineral acid, and shaken out with ether, the ether subsequently being washed with water to remove the mineral acid. The ethereal extract was then titrated with N/10 alkali in alcohol.

The unincubated required 0·8 c.c. = 0·0226 g. fatty acid.

The incubated required 3·1 c.c. = ·0874 g. fatty acid.

On subsequently boiling the extract of the incubated specimen with a measured amount of N/10 alcoholic potash, it was found that 5·8 c.c. were used up = 0·1709 g. of fat, which had been liberated. This fat scarcely contained any trace of phosphorus. Hence the alcohol liberated a certain amount of neutral fat, which had become ether-insoluble to direct ethereal extraction.

Other experiments gave similar results as regards the amount of fatty acids present in the ether extract of the second alcoholic extraction, and showed that neutral fat was also present which did not come out in the original extraction.

Exp. 2. *A mixture of chyle and blood treated as in Exp.* 1.

	Non-incubated	Incubated
Total residue	0·746 g.	0·402 g.
Neutral fat	0·5938 (20·2 c.c. N/10 alk.)	0·294 (10 c.c.)
Fatty acid	0·0	0·0
Lecithin	0·99	0·029
Cholesterin	0·068	0·073

Subsequent extraction as above.

Fatty acids	0·0328 g. (1·2 c.c.)	0·093 g. (3·3 c.c. alk.)
Fats	0·0	0·138 (4·7 ,,)

The same experiment was made with the same materials, but instead of subsequent alcoholic extraction the finely ground material after the ether extraction was digested with pepsin and hydrochloric acid and then dried and extracted with ether.

Exp. 3. Thus 100 c.c. of a mixture of blood and chyle were examined.

	(a) Unincubated	(b) Incubated
Primary extract	0·753 g.	0·449 g. (loss = 43·1 %)
Secondary ,,	0·028	0·32
Fatty acid in secondary extract	trace	0·0789
Neutral fat	nil	0·233 (7·9 N/10 alkali)

The same method as above was used. 200 c.c. of a mixture of blood and chyle were used for immediate estimation by ether extraction followed by treatment with pepsin and extraction, and two separate lots of 200 c.c. were incubated in an air current at 37° for 24 hours, one examined by ether-alcohol extraction, and divided into its constituents, the other first extracted with ether and then digested with pepsin and acid and again extracted with ether.

Exp. 4.

	Control	Specimen "A" ether-alcohol extraction	Specimen "B" digestion with pepsin, etc.
	g.	g.	g.
Total residue (first extract)	1·64	0·813 (loss = 50·4 %)	0·799 (51·8 %)
Neutral fats	1·3934 (47·2 c.c. N/10 alk.)	0·705 (23·9 c.c. N/10 alk.)	—
Fatty acids	0·0	0·0	—
Lecithin	0·198	0·087	—
Cholesterol etc. ...	0·0486	0·021	—
Fatty acid in second extract	0·0479 (1·7 c.c. N/10 alk.)	0·121 (4·3 c.c. N/10 alk.)	0·1325 (4·7 c.c.)
Fat in second extract ...	nil	0·545 (18·5 c.c.)	0·7139 (24·2 c.c.)

It was also found that by extracting with alcohol on the water-bath and then with ether, the whole of the ether-insoluble fat could be recovered.

Two lots of 100 c.c. of a mixture of chyle and blood were examined (a) unincubated, (b) incubated in the usual way.

	(a) Unincubated	(b) Incubated
Primary extract	0·712 g.	0·467 g.
Secondary „	0·028	0·298

These experiments further show that the neutral fats are not hydrolysed by the blood or chyle, but that the lecithin is the only fat hydrolysed by the ferments in the chyle or blood. This is at variance with the observation of Hamill [1907] who states that the chyle can hydrolyse a neutral fat, viz., olive oil.

(9) The question then arose if this ether-insoluble fat could be isolated and its constitution determined and especially if it were diffusible and soluble in water.

The method adopted was that of Cohnstein and Michaelis [1894], viz. diffusion through parchment thimbles into distilled water. In all the experiments it was found that only soaps, etc., diffused through, and no substance containing the neutral fat, and that the amount of fatty acid thus obtained corresponded to the degree of hydrolysis of the lecithins.

Experiments were also made with a lipanin emulsion, made with sterile ascitic fluid and lipanin. A very fine emulsion was produced.

It was found that on incubation with blood and a current of air the same results were obtained as above with chyle fat and that the fat could be recovered again by treatment with alcohol or pepsin and hydrochloric acid.

These observations are in accordance with those of Mansfeld [1907] who showed that the fat in lipanin after incubation with blood became non-extractable by ether, but could be recovered by Liebermann's method.

Hamburger [1900] was only able to obtain this result when he made his artificial chyle with a chyloid fluid. In these experiments, as in those of Mansfeld, ordinary ascitic fluid worked well.

Conclusions.

It would seem justifiable to draw the following conclusions:

1. Blood and chyle contain a ferment which can hydrolyse lecithin but not neutral fat.

2. When blood and chyle fat are incubated together, the neutral fat forms an absorption combination with the proteins and is thus rendered non-extractable by ether. The combination can, however, be broken up by peptic digestion, or treatment with alcohol. It is not formed during the process of drying.

3. The combination appears to be broken up by heat, since larger losses are obtained if the incubated mixtures are not heated, and if the material be dried at a low temperature.

.4. The formation of this complex appears to be due to the formed elements of the blood, since the corpuscles or material carried down with the corpuscles on centrifugalisation have the same effect, and serum by itself has not.

5. The action does not appear to be due to the haemoglobin, but to some material precipitated in the method of preparing the haemoglobin, since on rendering the haemoglobin inert as an oxygen carrier by carbon monoxide, the change still occurs.

6. The change occurs best in a current of air or oxygen, and if the mixture be thoroughly agitated.

7. The combination appears to be due to the action of a ferment because :

(*a*)   the haemoglobin may be rendered inert without affecting the process ;

(*b*)   ferment poisons seriously interfere with it.

8. Similar combinations may occur when blood is mixed with a very fine emulsion of fat. The compound, however, does not appear so stable since it is practically always broken up by heating and by alcohol.

9. The albumin-fat combination is not diffusible nor soluble in water. After incubation an increase in diffusible material takes place, but this is only due to the diffusion of the products of the lecithin hydrolysis, viz., glycerophosphoric acids, soaps, choline.

10. The chyle and corpuscles contain a ferment capable of breaking up lecithin and liberating the fatty acids.

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REFERENCES.

Arthus (1902), *J. Physiologie*, **4**, 56, 455.

Cohnstein and Michaelis (1894), *Pflüger's Arch.* **55–57**.

Fischler (1902), *Virch. Arch.* **170**, 100.

—— (1903), *Virch. Arch.* **174**, 34.

Hagemeister (1903), *Virch. Arch.* **172**, 72.

Hamburger (1900), *Arch. Anat. Physiol.* 544.

Hamill (1907), *J. Physiol.* **35**, 151

Hanriot (1896), *Compt. Rend. Biol.* **48**, 123, 925, 753.

Hepner (1898), *Pflüger's Arch.* **73**, 595.

Kastle and Loevenhart (1908), *Lancet*, July, 135.

Mansfeld (1907), *Centr. Physiol.* **21**, 666.

Moore, B. (1897), *Reports of the Johnston Lab.*, Liverpool.

Schultz (1897), *Pflüger's Arch.* **65**, 229.

Strauss (1907), *Deutsch. Med. Woch.* 37.

Weigert, R. (1900), *Pflüger's Arch.* **82**, 86.

XXIX. ON THE LIPOLYTIC ACTION OF THE TISSUES.

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The following experiments were carried out to test the lipolytic activity of the various organs towards the neutral fats.

It is only comparatively recently that such enzymes have been demonstrated in the tissues; their presence was inferred previously from the action of tissue extracts on simple esters, such as monobutyrin, etc. That these are by no means the same, has been shown by Cohnstein [1904] for the castor-oil seed. Arthus [1902] showed a similar difference for blood serum.

Nencki and Lüdy [1887] tested the lipolytic power of tissues on amyl salicylate and found that the tissue extracts worked best in an alkaline medium.

Hanriot [1896] used monobutyrin and demonstrated a lipolytic ferment in all tissue extracts except the thyroid. The enzyme ceased to act when a certain degree of acidity had developed and could continue when the acidity was neutralised. He also showed that the tissue extracts could hydrolyse the ethyl esters of formic, acetic, propionic and butyric acids.

Arthus [1902] drew attention to the errors in Hanriot's observations and was unable to confirm his work. Kastle and Loevenhart [1900] experimented with ethyl butyrate and found that the ferment was closely attached to the cells, and that liver extract was more active than the pancreatic on this ester, but that the pancreas could hydrolyse the esters of the higher fatty acids more readily than those of the lower.

Siebert [1900] studied the action of pancreatic extracts on egg yolk, and further, showed that the blood serum is incapable of splitting lecithin. Subsequently Umber and Brugsch [1906] experimented with tissue extracts, using egg yolk as their emulsion of neutral fat. The various organs were washed free from blood, then finely ground up with kieselguhr and the juice

pressed out with a hydraulic press. Two series of experiments were performed, one with the organs from an animal killed whilst digesting, the other in starvation. One series of experiments was conducted under aseptic conditions, the other under toluene.

These observers concluded that the tissue extracts can hydrolyse the fat in egg yolk and that during digestion the pancreatic extract is the most powerful, closely followed by that of the spleen, but that the liver and intestinal mucosa are more powerful than the pancreas. Further that activating bodies would appear to be present in some of the organs.

In the following series of experiments it was intended to study the action of the various tissue extracts on:

(1) Egg yolk.
(2) Artificial emulsions of lecithin.
(3) Lecithin not in a state of emulsion.
(4) Chyle obtained from the human subject.

The tissues were ground up finely in a mortar, mixed with an equal bulk of normal saline and pressed through a press so that the tissue extract consisted of a fine suspension of the tissue in normal saline. The tissues were removed under strict aseptic precautions; pigs' and dogs' organs were chiefly used. In the case of the pigs' organs, these were removed in the slaughterhouse in gauze soaked in 2·5 % carbolic, the outside was removed with a sterile knife and the tissue transferred to a sterile mortar and ground up with sterile normal saline. The dogs' tissues were obtained from healthy dogs, which were anaesthetised and bled to death. The animals were subsequently washed out with sterile normal saline. No toluene experiments are recorded, because it was found that even adding toluene up to 2 % did not inhibit the growth of organisms which were capable of splitting up egg yolk very readily. The "fat" containing emulsions were also prepared under strictly aseptic precautions and sterilised.

The mixtures of "fat" and tissue extract were incubated at 37° for 15–20 hours. At the end of that time they were examined directly and culturally for possible bacterial contamination and any showing this were rejected. The method of estimating the effect of the tissue extracts was to determine the total amount of "fat" present by boiling the mixture of unincubated material with sodium carbonate for some time on the water bath, and then, after acidulating with sulphuric acid, extracting with ether. The ethereal extract, after removal of the mineral acid, was then treated with N/10 KOH to neutralise the fatty acids, and then hydrolysed with alcoholic potash, evaporated, and taken up in water, acidulated with sulphuric acid

and the liberated fatty acids extracted with ether, which was then freed from sulphuric acid by shaking up with water. The ethereal solution of the fatty acids was titrated against N/10 alcoholic potash and then reckoned in terms of oleic acid; 1 c.c. N/10 KOH = 0·0282 g. oleic acid. The incubated mixtures were neutralised with sodium carbonate, well heated on the water bath and acidulated with sulphuric acid to liberate the fatty acids which were extracted with ether and purified as before. The amount of fatty acid was thus determined by titration with N/10 alcoholic potash.

In some of the experiments it became necessary to separate oleic acid and other high fatty acids from any lower acids, such as lactic acid, which might be formed from the carbohydrates. This was done by having a double series of mixtures; in the one the total fatty acids were determined in the way mentioned above, in the second the mixture, after neutralisation and boiling, was treated with sulphuric acid to liberate the fatty acids and then treated with ammonium sulphate, according to the methods of Magnus Levy, to separate the lower from the higher fatty acids.

A. *Effect of tissue extracts of various organs on sterile egg yolk mixture, containing 0·5 % sodium carbonate.*

10 c.c. tissue extract was used with 20 c.c. of yolk suspension. Results expressed in c.c. decinormal alkali.

No. of Exp.	Pigs' organs	Total fats as fatty acids	Fatty acids liberated	% hydro-lysed
1	Pancreas	53·2	48·0	90·0
	Liver	55·8	20·5	36·7
	Spleen	53·8	20·2	37·5
	Pancreas + 5 c.c. Spleen ...	53·4	40·0	75·0
	,, ,, ,, (the spleen was first boiled) ...	53·4	40·0	75·0
	Liver + 5 c.c. Spleen	56·0	21·6	38·6
	Pancreas + 5 c.c. Spleen ...	54·2	38·6	70·1
	Serum	Not determined	0·0	0·0
2	Pancreas	53·6	30·6	57·0
	10 c.c. Bile	51·8	19·08	36·7
	Pancreas + 5 c.c. Bile	54·0	30·0	55·5
	Pancreas + 5 c.c. Bile + 5 c.c. Serum	54·6	30·8	56·0
	10 c.c. Serum	Not determined	0·0	0·0
3	Pancreas	54·0	37·2	68·2
	Liver	55·2	21·8	39·5
	Kidney	54·6	20·4	37·87
	Muscle	54·4	20·2	37·0
	Spleen	54·4	21·6	40·0
	Pancreas + 5 c.c. Spleen ...	54·8	39·1	71·0
	Muscle + 5 c.c. Spleen ...	54·4	21·0	39·87

Dogs' Tissues.

5 c.c. of extract + 20 c.c. of yolk suspension in each experiment.   Results expressed as before.

		Total fats	Fatty acids	% hydrolysed
Dog 1	Spleen	56·1	20·5	36·5
Hungry	Liver	56·8	20·5	36·0
animal	Pancreas	55·9	22·8	40·9
	Kidney	56·3	20·2	35·8
	Liver + Spleen (5 c.c. of each) ...	57·1	22·0	38·0
	Pancreas + Spleen (5 c.c. of each)	56·2	37·0	65·8
	Pancreas + 5 c.c. of Serum ...	58·4	37·5	66·2

5 c.c. of extract + 20 c.c. of yolk suspension in each experiment.   Results expressed as before.

		Total fats	Fatty acids	% hydrolysed
Dog 2	Pancreas	54·2	41·0	75·5
Digesting	Pancreas + Serum	54·4	37·0	68·0
animal	Serum	—	0·0	0·0
	Liver	55·0	20·8	37·8
	Spleen	54·3	20·2	37·0
	Pancreas + Spleen	54·6	44·5	81·5
	Pancreas + boiled Spleen ...	54·6	41·5	77·0

5 c.c. of extract + 20 c.c. of yolk suspension in each experiment.   Results expressed as before.

		Total fats	Fatty acids	% hydrolysed
Dog 3	Pancreas	52·8	30·0	57·0
Hungry	Pancreas + Liver (5 c.c. of each)	53·6	34·0	63·4
animal	Pancreas + Spleen (5 c.c. of each)	53·2	33·8	61·2
	Spleen	53·9	21·2	40·0
	Liver	53·4	22·0	40·1

Human Tissues.

Removed twelve hours after death.   20 c.c. of egg yolk and 10 c.c. of the tissue suspension. Results expressed as before.

	Total fats as fatty acids	Fatty acids liberated	% liberated
Pancreas	56·2	46·0	81·8
Pancreas and Liver (5 c.c.) ...	57·0	48·0	84·3
Pancreas and boiled Liver (5 c.c.)	57·0	49·0	85·9
Liver	56·8	21·0	37·0
Spleen	56·0	21·4	38·2
Liver + 5 c.c. Spleen	57·0	23·0	40·3
Pancreas + 5 c.c. Spleen... ...	56·6	48·6	85·6

Sheep's Tissues.   Quantities as before.

Liver	54·8	20·2	36·6
Spleen	54·0	19·8	37·0
Liver + Spleen (5 c.c.) ...	55·0	20·4	37·0
Liver + boiled Spleen (5 c.c.) ...	55·2	20·4	37·0
Pancreas	53·8	30·2	56·4
Pancreas + 5 c.c. Spleen... ...	54·2	32·0	58·6

From these experiments it will be seen that the tissues have the power of breaking up the lecithin when it is exhibited to them in the form of egg yolk emulsion.

In all cases the pancreas has a much more powerful action than any other tissue extract, and there is from these experiments no distinct evidence of

any kinase capable of augmenting the activity of the ferments. The only example of this effect occurs in one of the experiments with the tissues of a dog in a fasting state, but in the other experiments with the organs of a dog, and with those of pigs and sheep, which are always slaughtered in a fasting condition, there is absolutely no evidence of any activating ferment in any of the tissues. In these respects these experiments do not agree with those of Umber and Brugsch [1906].

The above results, however, like those of Umber and Brugsch, take no notice of the formation of the lower fatty acids, which Magnus Levy [1902] showed were formed during the incubation of tissues from the carbohydrates present. In order, therefore, to eliminate this error, the following experiments were performed in double series; in one the total fatty acids were estimated, in the other the lower fatty acids were estimated after separation by ammonium sulphate, the difference between the two being due to the amount of fatty acid liberated by the hydrolysis of the fat.

In the following series of experiments 10 c.c. of tissue juice were incubated with 20 c.c. of yolk suspension in 0·5 % sodium carbonate at 37° for 18 hours. The results here are in terms of N/10 NaOH as before.

No. of Exp.	Pigs'	Total fatty acids found	Lower fatty acids	Higher fatty acids	Total fat in terms of fatty acids	% hydro-lysed
1	Liver	23·0 c.c.	7·6 c.c.	15·4 c.c.	52·0	29·6
	Spleen	21·0	3·2	17·8	51·2	34·7
	Kidney	18·0	4·0	14·0	51·3	27·3
	Pancreas	39·0	1·5	37·5	51·0	73·5
2	Sheep's					
	Pancreas	31·0 c.c.	2·0 c.c.	29·0 c.c.	51·0	56·8
	Liver	19·0	6·1	12·9	51·8	27·9
	Spleen	12·8	1·8	11·0	51·2	21·5
	Kidney	13·2	4·0	9·2	51·4	17·9
3	Dogs'					
	Pancreas	38·0 c.c.	1·4 c.c.	36·6 c.c.	49·2	74·4
	Liver	18·0	4·1	13·9	49·8	27·8
	Spleen	17·0	5·7	11·3	49·0	23·0
	Muscle	12·0	5·2	6·8	49·3	13·7

These experiments do not very greatly alter the results of the previous experiments, and confirm the lipolytic power of the various tissue extracts towards egg yolk emulsion.

B. *Experiments with liver extracts on lecithin, not in a state of emulsion.*

Weighed quantities of lecithin were placed in sterile flasks and sterilised. Then sterile tissue extract was added and allowed to act for varying intervals in the cold or warm.

In Exp. 1.   19 % was broken up. ⎫ In 16 hours at 37°.
    ,,   2.   23 %   ,,   ,,   ⎭
    ,,   3.   18 %   ,,   ,,   In 14 days at 4°.
    ,,   4.   56 %   ,,   ,,   ,, 28 ,,   ,,

The result in each case should be somewhat higher, because no account was taken of the amount of lecithin in the liver extract added. It is interesting to note that jecorin could also be found in all these experiments after incubation.

C. *Action of tissue extracts on chyle.*

The chyle was obtained from a healthy young man suffering from lymphatic obstruction, producing lymphangiectasis in one lower limb. The chyle could be obtained under aseptic conditions in large quantities after a period of blocking the fistulae in the lower limb. It contained from 1·3 to 1·6 g. of fat per cent., and from 0·064 to 0·08 g. of lecithin.

The lipolytic ferment in the chyle was destroyed by heating to 60° for an hour, thus also sterilising the fluid.

Sodium carbonate was added to make a 0·5 % solution.

In these experiments a double series was made in order to determine the relative amounts of higher and lower fatty acids.

20 c.c. of extract of the finely pounded tissue were added to 50 c.c. of chyle and incubated for 18 hours at 37°. Results expressed in c.c. of $N/10$ NaOH.

No. of Exp.	Pigs'	Total fats	Fatty acids found	Lower fatty acids	Higher fatty acids	% hydrolysed
1	Pancreas	24·0	18·8	1·6	17·2	71·5
	Liver	27·2	13·0	6·4	6·6	24·2
	Spleen	23·1	5·6	2·6	3·0	13·0
	Kidney	24·8	9·4	4·2	5·2	21·0
2	Pigs'					
	Pancreas	22·9	14·2	0·8	13·4	58·5
	Liver	25·8	9·2	3·8	5·4	20·9
	Spleen	23·2	7·4	4·8	2·6	11·2
	Kidney	24·4	6·8	3·0	3·8	15·6
3	Sheep's					
	Pancreas	22·0	13·2	1·0	12·2	55·4
	Liver	27·1	9·2	4·0	5·2	18·0
	Spleen	22·4	5·6	3·2	2·4	10·7
	Kidney	25·0	6·0	2·0	4·0	16·0
4	Dogs'					
	Pancreas(10c.c.)	21·6	15·0	1·0	14·0	65·0
	Liver	30·3	13·4	4·2	9·2	30·3
	Spleen	24·7	6·5	2·8	3·7	15·0
	Kidney	26·6	8·2	3·1	5·1	20·0

These results show that a certain amount of the fatty acids found in the

incubated mixture is due to the formation of lower fatty acids. Further, that the tissues have the power of liberating higher fatty acids when incubated with chyle, but as chyle and the tissues contain not only neutral fat, but lecithin as well, the question then arises as to the relative proportion hydrolysed by the tissue ferments. Finally the figures are quite small when compared with those obtained by using egg yolk.

To determine this question a series of experiments was carried out in which the lecithin, the total amount of fats and the higher fatty acids formed were determined before and after incubation.

For this purpose three sets of each mixture were made. The lecithin was determined by extracting the material with alcohol and ether, evaporating, taking up in ether, precipitating by acetone and determining the phosphorus in the precipitate by Neumann's method.

1 c.c. N/2 NaOH = 0·014331 g. of lecithin.

Lecithin contains 66 % fatty acids.

Quantities as before. Results reckoned as fatty acids in terms of N/10 alkali.

Exp. 1. Pigs'

	Liver	Pancreas	Spleen
Total fats	25·1 c.c.	21·1 c.c.	23·5 c.c.
Lecithin	0·261 g.	0·0902 g.	0·1504 g.
Lecithin fatty acids ...	6·1 c.c.	2·1 c.c.	3·5 c.c.
Fatty acids liberated ...	10·1	14·0	6·8
Lower fatty acids liberated	4·2	1·8	3·0
Higher ,, ,,	5·9	12·2	3·8
Lecithin left	nil	nil	nil
Fatty acid in Lecithin ...	6·1	2·1	3·5
Fatty acid found ...	5·9	12·2	3·8

Exp. 2. Dog's Liver. Quantities as before.

Total fats	25·9 c.c.
Lecithin	0·339 g.
Lecithin fatty acids	7·9 c.c.
Fatty acids liberated	12·2
Lower fatty acids liberated	4·0
Higher ,, ,, ...	8·2
Lecithin left	nil

Fatty acids in the lecithin = 7·9 c.c. N/10 NaOH.
,, ,, found liberated = 8·2 ,, ,,

Exp. 3. Quantities as before.

Sheep's

	Liver	Spleen	Kidney
Total fats as fatty acids ...	30·4 c.c.	28·9 c.c.	30·2 c.c.
Lecithin	0·244 g.	0·177 g.	0·235 g.
Fatty acids in Lecithins ...	5·7 c.c.	4·2 c.c.	5·5 c.c.
Fatty acids liberated ...	11·3	8·4	7·8
Higher fatty acids liberated	5·5	3·9	5·3
Lower ,, ,,	5·8	4·5	2·5
Lecithin left ,,	nil	nil	nil

Exp. 4. Pigs'	Liver	Kidney	Spleen	Spleen + Liver (10 c.c. of each)
Total fats	26·8 c.c.	25·4 c.c.	24·2 c.c.	21·1 c.c.
Lecithin	0·2178 g.	0·1576 g.	0·1089 g.	0·3152 g.
Fatty acid in lecithin ...	5·1 c.c.	3·7 c.c.	2·6 c.c.	7·4 c.c.
Fatty acids liberated ...	8·3	5·2	6·5	13·7
Lower fatty acids liberated	2·8	2·1	4·0	6·1
Higher ,, ,,	5·5	3·1	2·5	7·6
Lecithin	nil	trace	nil	nil

In all these experiments it will be noticed, with the exception of that with pancreatic extract, that the amount of fatty acids liberated corresponds to the amount of fatty acids present in the lecithin, and that in all cases the lecithin is practically completely hydrolysed.

Evidence of the inability of the lipase of the liver to attack neutral fat is obtained from experiments on aseptic autolysis at 4°. Fat expressed as c.c. N/10 NaOH %.

Duration of autolysis (days)	Dogs' Tissues	Sheep's Tissues
0	16·2	21·0
7	16·6	20·4
21	15·8	21·6
42	16·2	22·4
91	16·8	—
168	—	22·8
224	—	22·4

This shows that ordinary fat is not hydrolysed, but that lecithin is hydrolysed at 4°. These results indicate that the liver, spleen and kidney contain a lipolytic ferment which attacks lecithin quite readily, but has no apparent action on the simple glycerides of the higher fatty acids even when presented in the form of the finest emulsion as in chyle, or as they exist in the tissue itself as is seen in the autolytic experiments.

It is possible that the reason of this lies in the fact that lecithin can form in water a colloid suspension, and so can be more readily attacked, but the previous results show that lecithin can be readily split up even when presented to the tissue extracts in a very coarse form. The other and more probable explanation would appear to be that when the glycerides of the higher fatty acids are taken to the liver or other tissues they are converted into lecithins and stored up free or in combination with the protein as the invisible unstainable fat in the normal organ and in this form are easily hydrolysed by the lipolytic ferment as required. This view seems the more probable from the results of Leathes and Kennaway [1909], who showed that the liver desaturates fats brought from the food or fat depots; this agrees with the high iodine values of the lecithins, showing that the lecithins are

formed from these desaturated fatty acids. Further, it is important to note in the quantitative estimations of the "fats" in the liver, etc., that by far the greatest amount is accounted for by the lecithins, nearly 90 %.

D. *Experiments to ascertain the effect of reaction on the lipolytic power of the organs.*

In each of these a double series of experiments was performed to ascertain the amount of higher fatty acids.

Pig's Liver.   10 c.c. of extract with 20 c.c. of egg yolk suspension.

1. *Effect of Alkali.*

With g. Na$_2$CO$_3$	(%)	Total fatty acids	Lower	Higher	Total fat	% hydrolysed
0·05	(0·16)	14·4	- 6·0	8·4	40·8	20·7
0·1	(0·32)	21·6	7·6	14·0	40·8	34·3
0·15	(0·5)	23·2	7·4	15·8	40·8	38·7
0·2	(0·66)	18·2	6·1	12·1	40·8	29·6
0·3	(1·0)	10·1	4·0	6·1	,,	14·9
0·4	(1·33)	6·0	2·1	3·9	,,	9·0

Same quantities as above, but 30 c.c. water added.

0·05	(0·08)	12·5	4·6	7·9	40·2	19·6
0·1	(0·16)	20·0	6·0	14·0	,,	34·8
0·15	(0·24)	21·4	6·6	14·8	,,	36·8
0·2	(0·33)	16·2	4·6	11·8	,,	29·3
0·3	(0·5)	9·3	2·4	5·9	,,	14·6
0·4	(0·66)	5·0	2·0	5·0	,,	7·4

Thus it will be seen that the activity does not depend on the percentage of alkali present, but on the total amount.

2. *Effect of lactic acid.* The quantities used were as before. Incubation 16 hours. The amount of lactic acid is in c.c. of the decinormal solution.

Dog's Liver.   10 c.c. of extract with 20 c.c. of egg yolk solution and all made up to 40 c.c.

C.c. N/10 acid	Total fats c.c. N/10 alk	Total fatty acids found	Lower	Higher	% hydrolysed
2	48·2	20·2	6·8	13·4	27·8
4	,,	23·8	7·9	15·9	32·9
6	,,	24·9	9·3	15·6	32·6
8	,,	23·1	12·4	10·7	22·2
10	,,	20·8	13·0	7·8	16·2

Pig's Liver.   Quantities as above.

2	48·2	18·6	8·1	10·5	21·8
4	,,	23·8	9·7	14·1	29·2
6	,,	24·2	10·4	9·8	20·3
8	,,	21·2	11·8	9·4	19·5
10	,,	19·8	14·1	5·7	11·8

SUMMARY.

From these experiments we can conclude that:

(1)   The tissues possess a true lipolytic ferment.

(2)   The lipolytic ferment, with the exception of the pancreatic lipolytic ferment, can only hydrolyse phosphatides and jecorins, but not ordinary fats.

(3)   The ferment is capable of acting in an alkaline or acid medium.

(4)   There is no evidence of a kinase in the spleen.

REFERENCES.

Arthus (1902), *J. Physiologie*, **4**, 56.

Cohnstein (1904), *Ergebnisse der Physiologie*, **3**, 194.

Hanriot (1896), *Compt. Rend.* **123**, 753, 833.

Kastle and Loevenhart (1900), *Amer. Chem. J.* **24**, 491.

Leathes and Kennaway (1909), *Lancet*, 95.

Magnus Levy (1902), *Beiträge*, **2**, 201.

Nencki and Lüdy (1887), *Therap. Monatsheft*, 417.

Siebert (1900), *Zeitsch. physiol. Chemie*, **49**, 50.

Slade (1903), *Beiträge*, **3**, 291.

Umber and Brugsch (1906), *Arch. Exp. Path.* **55**, 164.

XXX. THE ESTIMATION OF TYROSINE IN PROTEINS BY BROMINATION.

By ROBERT HENRY ADERS PLIMMER and
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The tyrosine content of a protein is usually ascertained by hydrolysing the protein with sulphuric acid, removing the acid as barium sulphate, concentrating the solution and washings and then weighing the tyrosine which separates out. The data so obtained by various workers are in some cases very concordant, but it is generally considered that the real tyrosine content of a protein is higher than these figures represent since it is frequently impossible to isolate the whole of the tyrosine. Osborne and Clapp [1906] and Osborne and Guest [1911] have particularly emphasised this point and they consider that the tyrosine content of a protein so obtained is only a minimal one, the true content being from one half to one per cent. higher. The presence of cystine in the tyrosine isolated has also never been taken into consideration. The two substances are so alike in their solubility in water, in acids and in alkalies that their separation is a matter of considerable difficulty [Plimmer, 1913]. Losses occurring in the isolation of the tyrosine may be compensated for by admixture with cystine.

It was shown by J. H. Millar [1903] that tyrosine was readily brominated and converted into dibromotyrosine, and that the amount of tyrosine in a simple mixture of amino-acids could be accurately estimated by means of this reaction.

A. J. Brown and E. T. Millar [1906] using this reaction showed that the tyrosine was completely liberated at a very early stage in the hydrolysis of edestin by trypsin. Their data gave the tyrosine content of edestin as 4·06 per cent., a figure which is considerably higher than that obtained by direct isolation (2·1 per cent.). They made no estimations of the tyrosine

content of other proteins. The higher value agrees with Osborne's supposition that the tyrosine content of proteins as obtained by isolation is only a minimal one and it seemed very desirable that further estimations should be made by this method.

It was found that it was better to alter Millar's method as it was not sufficiently delicate for estimating small amounts of tyrosine such as are obtained by the hydrolysis of proteins. The procedure of Brown and Millar also involved a large deduction for the amount of bromine absorbed by the control (protein or other decomposition products, possibly histidine) which prevented an accurate estimation. The absorption of bromine by the unchanged protein and its product of hydrolysis, histidine, which according to Knoop [1908] reacts with bromine, has been eliminated by the use of phosphotungstic acid. Another disturbing factor is the presence of tryptophane with the tyrosine amongst the products of hydrolysis. Though tryptophane may be destroyed by boiling with acids, its decomposition products still absorb bromine. The estimation of tyrosine amongst the products of the acid hydrolysis of proteins was therefore impossible. It remained to take advantage of the rapid and complete liberation of tyrosine during the early stages of tryptic digestion and the slower liberation of tryptophane, which is only complete after several days when a moderately active trypsin solution is employed [Hopkins and Cole, 1901]. By estimating the bromine absorption after short intervals of digestion in the filtrate from the phosphotungstic acid precipitate, values for the tyrosine content of proteins have been obtained which agree closely with those found by direct isolation.

Our work was withheld since we felt considerable diffidence as to the exactness of our data, but since the publication of much higher figures for the tyrosine content of proteins by Folin and Denis [1912] by a new colorimetric method and since these figures have been criticised by Abderhalden and Fuchs [1913] our data are of some value in support of the results of Abderhalden and Fuchs. Folin and Denis do not seem to have taken sufficient account of the presence of tryptophane and oxytryptophane, which react with their reagent. If their figures express the total tryptophane and tyrosine content, and our values the tyrosine content alone, then the difference will give the tryptophane content of proteins which is at present unknown and of considerable importance.

EXPERIMENTAL.

(1) *The Estimation of Small Amounts of Pure Tyrosine by Bromination.*

J. H. Millar's method for the estimation of tyrosine depends upon the formation of dibromotyrosine when tyrosine is treated with nascent bromine; this is liberated by adding sodium bromate to an acid solution of potassium bromide. The equations representing the reaction are:

$$NaBrO_3 + 5KBr + 6HCl = NaCl + 5KCl + 3Br_2 + 3H_2O,$$
$$C_6H_4(OH).CH_2.CH(NH_2).COOH + 2Br_2$$
$$= C_6H_2Br_2(OH).CH_2.CH(NH_2).COOH + 2HBr,$$
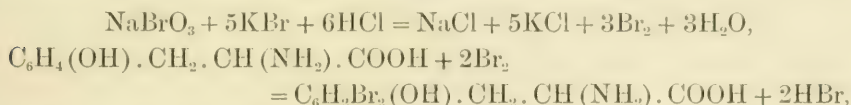
from which we find that 1 g. of tyrosine absorbs 1·765 g. of bromine and corresponds to 0·5558 g. of sodium bromate.

His procedure was to add 10–15 cc. of a 20 per cent. solution of potassium bromide to a solution of tyrosine in hydrochloric acid and to titrate with M/5 sodium bromate solution until it assumed a persistent deep yellow colour. If the solution were coloured, starch and potassium iodide were used as an indicator.

Millar found that 1·808 g. of bromine were absorbed by 1 g. of tyrosine, a figure which is slightly higher than the theoretical value but of sufficient accuracy to show that tyrosine can be estimated in solution by bromination; in one of the experiments 0·2 g. tyrosine required 3·8 cc. of the bromate solution.

The estimation of smaller amounts of tyrosine than 0·2 g. was not investigated by J. H. Millar. The amount of tyrosine in a protein does not usually exceed 3 per cent. except in the case of caseinogen which contains from 4·5–5 per cent. and silk-fibroin which contains about 10·5 per cent. One gram of protein would therefore usually give a solution containing 0·01–0·04 g. tyrosine. To estimate 0·01 g. tyrosine, 0·19 cc. of M/5 bromate solution would be required and an error of 0·05 cc. in the titration would make an error of 25 per cent. in the estimation. It was therefore necessary to ascertain if tyrosine could be brominated by a more dilute solution of sodium bromate. Estimations were therefore made with an N/5 (= M/30) solution of bromate in the same way as described by J. H. Millar, thus:

0·1911 g. tyrosine in 25 cc. hydrochloric acid solution required

21·05 cc. M/30 bromate solution;

i.e. 1 g. tyrosine absorbs 1·75 g. bromine (theoretical 1·765).

The bromination of tyrosine by the M/30 solution of sodium bromate is slower than by the M/5 solution and it appeared that the method might be more convenient if the bromination were effected by adding excess of the sodium bromate solution to the tyrosine solution, allowing the reaction to proceed for 10–15 minutes in a closed flask, adding potassium iodide and titrating the excess of halogen with thiosulphate solution using starch as indicator.   2 g. of tyrosine were dissolved in 500 cc. hydrochloric acid;

50 cc. + 30 cc. M/30 NaBrO$_3$ solution; 7·1 cc. thiosulphate solution; 22·9 cc. bromate required.

50 cc. + 30 cc. M/30 NaBrO$_3$ solution; 7·2 cc. thiosulphate solution; 22·8 cc. bromate required.

i.e. 50 cc. contain 0·207 g. tyrosine.

The absorption of bromine for 1 g. of tyrosine is 1·825 g., a value slightly higher than the theoretical (1·765), but comparable with that found by J. H. Millar (1·808). In subsequent experiments this high figure was not obtained. It seems to have been due to the presence of a rather large excess of bromate solution; the vapour in the flask appeared yellow and loss occurred during the titration.

Small quantities of tyrosine can thus be accurately estimated by making this alteration in the procedure.

(2)   Estimation of Tyrosine in the Presence of Protein and its Products of Hydrolysis.

It has been shown by A. J. Brown and E. T. Millar that proteins absorb bromine under the conditions employed for the estimation of tyrosine by J. H. Millar's method, but this did not preclude the estimation of tyrosine in the presence of unaltered protein if the amount of bromine absorbed by the protein was deducted from the amount absorbed by the mixture of protein and tyrosine. They showed further that there was no increase in the absorption of bromine by gelatin during its digestion by trypsin. Gelatin does not contain tyrosine or tryptophane, and since it was found that trypto-phane did not absorb bromine under the same conditions it was concluded that the increase in the absorption of bromine when edestin was digested by trypsin was entirely due to the liberation of tyrosine. The absorption of bromine due to the separation of tyrosine increased very rapidly and reached a maximum in about one hour. The result gave the tyrosine content of

edestin as 4·06 per cent. which differs very greatly from the value (2·1 per cent.) obtained by the isolation and weighing of tyrosine.

An examination was made for possible sources of error occurring during the estimation of tyrosine by bromination.

Brown and Millar used M/5 sodium bromate solution and 50 cc. of a 1 per cent. solution of edestin.

0·80 cc. of the bromate solution was required for the bromination and from this 0·42 cc. was deducted for the control. The difference of 0·38 cc. gave the tyrosine content.

As shown above an error of 0·05 cc. in the titration, if M/5 sodium bromate solution be used, corresponds to a difference of 25 per cent. in the amount of tyrosine when estimating 0·01 g. of tyrosine, which is equivalent to a variation of 1 per cent. in the tyrosine content of edestin. A proportionate error must be considered when the deduction for the control is made. The error in titration can be reduced by employing M/30 bromate solution. An error of 0·23 cc. in the titration will now correspond to a difference of 10 per cent. in the amount of tyrosine, which is equivalent to a variation of 0·4 per cent. for the tyrosine content of edestin. Dilution of the reagent will thus reduce the error but it is not in itself sufficient to make the method an accurate one.

The amount which has to be deducted for the control is greater than the amount used in the actual estimation; greater accuracy can therefore only be obtained by reducing or eliminating this deduction.

The constituents of a protein which are known to absorb bromine are tyrosine, tryptophane and histidine [Knoop, 1908]. According to Brown and Millar tryptophane does not absorb bromine under the conditions existing during the estimation of tyrosine so that it is most probably the histidine which absorbs the bromine and necessitates the large deduction for the control. Histidine can be removed by precipitation with phosphotungstic acid which leaves the tyrosine (and tryptophane) in solution in the filtrate. Hence if the deduction for the control be due to the presence of histidine, the absorption of bromine by the filtrate should be due solely to the tyrosine contained in the enzyme preparation. A preliminary experiment with gelatin showed that this was the only deduction necessary.

A 2 per cent. solution of trypsin was allowed to digest in the presence of chloroform; at intervals 20 cc. were removed and placed in 50 cc. of 5 per cent. sulphuric acid + 20 cc. of 10 per cent. phosphotungstic acid. To 50 cc. filtrate (= 0·333 g. trypsin) 10 cc. of sodium bromide (2 per cent.) and 10 cc. of the sodium bromate solution (1 cc. = 0·012 g. Br) were added;

after 10–15 minutes 10 cc. sodium iodide (4 per cent. solution) were added and the mixture titrated with sodium thiosulphate solution (1 cc. = 0·00663 g. Br) using starch as indicator[1].

Time	Thiosulphate required	Bromine absorbed
0	15·1 cc.	0·02 g.
4 hours	11·1	0·046
6 ,,	11·8	0·042
24 ,,	12·1	0·040

Taking the absorption after 6 hours, 1 g. of trypsin absorbs 0·126 g. bromine.

A 2 per cent. solution of gelatin in 0·4 per cent. sodium carbonate solution was digested in the presence of chloroform with 0·2 per cent. trypsin. 50 cc. samples were removed at intervals and placed in 50 cc. of 5 per cent. sulphuric acid + 20 cc. of 10 per cent. phosphotungstic acid. 100 cc. of the filtrate (= 0·833 g. gelatin) were treated with 10 cc. sodium bromide solution + 10 cc. sodium bromate solution (1 cc. = 0·012 g. bromine) and after half an hour the mixture was titrated with sodium thiosulphate (1 cc. = 0·00663 g. bromine) after adding sodium iodide and starch as indicator. Each sample contains 0·833 g. trypsin.

Time	Thiosulphate required	Bromine absorbed	Bromine absorbed by 0·0833 g. trypsin	Bromine absorbed by gelatin
0	17·4 cc.	0·0046	0·0025	0·0021
4 hours	16·2	0·0126	0·0104	0·0022
6 ,,	16·25	0·0126	0·0104	0·0022
24 ,,	15·6	—	—	—

The slight absorption by the gelatin (= 0·24 per cent.) is most probably due to the presence of tyrosine; the sample (gold label) gave a distinct reaction with Millon's reagent.

This procedure was then applied to the estimation of the tyrosine in caseinogen.

100 cc. samples of a 1 per cent. caseinogen solution were digested with 5 cc. of a trypsin solution for periods of 1–5 hours and were then precipitated with 25 cc. phosphotungstic acid solution in hydrochloric acid; 50 cc. of the filtrate were used for the titration:

[1] In the presence of phosphotungstic acid it is better to use sodium bromide instead of potassium bromide and sodium iodide instead of potassium iodide as potassium phosphotungstate is precipitated when potassium salts are present and the precipitate interferes with the titration with thiosulphate when starch is used as indicator. Auld and Mosscrop [1913] have maintained that starch and potassium iodide cannot be used when estimating tyrosine in digests of protein by the Millar method. Colourless filtrates are obtained after precipitation with phosphotungstic acid and with the alteration in the procedure the disappearance of the blue colour when the solution is titrated with thiosulphate is quite sharp. No difficulty has been experienced in determining the end point under these conditions.

Time (hours)	M/30 bromate absorbed	Difference
0	2·4 cc.	—
1	3·8	1·4
2	4·1	1·7
3	4·6	2·2
4	4·6	2·2
5	4·55	2·15

The maximum absorption of bromine occurred after 3 hours and then remained constant. The percentage of tyrosine in caseinogen calculated from the above difference is 5·08, a figure which agrees well with those usually given (4·5–5).

The following duplicate experiments with another solution of caseinogen show the reliability of the procedure[1].

	(1)				(2)		
Time (hrs.)	Bromate added	Thiosulphate required	Bromate absorbed		Bromate added	Thiosulphate required	Bromate absorbed
0	5 cc.	9·65 cc.	0·2 cc.		5 cc.	9·55 cc.	0·25 cc.
0·5	,,	7·45	1·3		,,	7·3	1·35
1	,,	5·95	2·05		,,	5·9	2·05
2	,,	4·8	2·6		,,	4·85	2·6
3	,,	4·35	2·85		,,	4·35	2·85

The estimation of tyrosine in the phosphotungstic acid filtrate is thus possible if no other products which absorb bromine are present. Gelatin does not contain tryptophane and cystine and it only contains a small amount of phenylalanine. Cystine and phenylalanine have been found not to absorb bromine, but the behaviour of tryptophane, which according to Brown and Millar does not react with bromine under the conditions adopted by J. H. Millar, probably because of the presence of hydrochloric acid, required further investigation as the method had been modified and as Dr Hopkins had informed us that tryptophane did react with bromine when treated in this way. A quantity of tryptophane was kindly supplied to us by Dr Hopkins for this purpose and the following experiments were carried out:

0·0631 g. tryptophane was dissolved in hydrochloric acid and titrated directly with sodium bromate by Millar's method.

7·3 cc. bromate were required. Bromine absorbed = 0·112 g.

0·0238 g. tryptophane was dissolved in hydrochloric acid: 10 cc. sodium bromide and 10 cc. sodium bromate solution (= ·1536 g. Br) were added: after

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[1] These experiments were carried out by one of us in conjunction with Mr S. H. Wood and the results were communicated to a meeting of the Physiological Society in March 1907. Continuation of the work was not then possible and no further experiments were made until 1909. The results of these later experiments were communicated to the Biochemical Club in March 1912.

15 minutes titrated with sodium thiosulphate solution (1 cc. = 0·00663 g. Br) after adding sodium iodide and starch : 3·2 cc. were required. Bromine absorbed = 0·1321 g.

0·0200 tryptophane was treated as in the previous experiment : 6·4 cc. thiosulphate were required. Bromine absorbed = 0·1105 g.

0·57 g. tryptophane was dissolved in 1000 cc. water; 100 cc. of this solution were used in each of the following experiments :

(1 cc. NaBrO₃ = 0·012 g. Br and 1 cc. thiosulphate = 0·00663 g. Br.)

	HCl	NaBr	NaBrO₃	Titrated after	Thiosulphate required	Bromine absorbed
100 cc.	5 cc.	10 cc.	10 cc.	15 mins.	2·05 cc.	0·1064 g.
	10	10	10	,,	1·40	0·1107
	1·5	10	10	,,	2·30	0·1048
	5	10	20	,,	17·95	0·1210
	10	10	20	30 mins.	17·0	0·1270
	5	10	20	,,	16·5	0·1306
	10	10	20	1·5 hrs.	15·05	0·1400

Another series of experiments gave similar results.

Tryptophane thus absorbs bromine under the conditions adopted by Millar; it absorbs a greater amount of bromine under the modified conditions, the absorption increasing with a larger amount of bromate and with the time allowed for the reaction. The absorption corresponds to about 6 atoms of bromine by 1 molecule of tryptophane.

The presence of tryptophane in solution with tyrosine will thus interfere with the estimation of tyrosine.

According to Hopkins and Cole tryptophane is destroyed by prolonged boiling with acids. If its products of decomposition do not absorb bromine the estimation of tyrosine should be possible after acid hydrolysis. Some experiments were therefore made to see if tryptophane still absorbed bromine after boiling with acids :

100 cc. of the above solution of tryptophane were heated for 10 hours with excess of concentrated hydrochloric acid (50 cc.). 10 cc. NaBr + 20 cc. NaBrO₃ were added and after 15 minutes titrated with thiosulphate (16·5 cc. required). Bromine absorbed = 0·110 g.

0·058 g. tryptophane was dissolved in 100 cc. water : 10 cc. were titrated directly : 10 cc. after boiling with concentrated hydrochloric acid and 10 cc. after boiling with 25 per cent. sulphuric acid. The bromine absorbed was respectively 0·0361 g., 0·0057 g. and 0·0106 g.

0·0998 g. tryptophane was dissolved in 50 cc. water : 10 cc. were titrated directly and 10 cc. after boiling for 5 hours with hydrochloric acid. The bromine absorptions were respectively 0·1017 g. and 0·074 g.

Absorption of bromine still occurs, but to a less extent, after boiling tryptophane with acid, so that the estimation of tyrosine is not possible after the hydrolysis of protein by acids.

Brown and Millar have shown that the whole of the tyrosine is very rapidly liberated by the action of trypsin and our preliminary experiments with trypsin and caseinogen have confirmed their results. It has been shown by Hopkins and Cole that the liberation of tryptophane does not occur rapidly with moderately active trypsin solutions and that its amount in solution only reaches a maximum after several days. This difference in the rate of liberation of the two substances may therefore permit of the estimation of the tyrosine content of a protein when the bromine absorption is measured at intervals during the digestion and when only those amounts absorbed in the early stages, from 6–24 hours, are taken as a measure of the tyrosine content.

(3) *Absorption of Bromine during the Tryptic Digestion of Proteins.*

Specimens of several animal and vegetable proteins have been procured and examined by the method described above. The vegetable proteins were most kindly sent to us by Prof. Osborne, the specimens in most cases being the same as those in which he and his co-workers had determined the tyrosine content by direct isolation and weighing.

In general, a 1 per cent. solution of the protein in 0·25 per cent. sodium carbonate was digested in the presence of chloroform or carbon tetrachloride with a 0·1 per cent. solution of trypsin. 50 cc. samples were removed immediately and after various intervals of time and then precipitated with 100 cc. of 10 per cent. phosphotungstic acid in 5 per cent. sulphuric acid. 100 cc. of the filtrate were then taken for the estimation. 10 cc. sodium bromide (2 per cent.) and 10 cc. sodium bromate solution were added; after 15 minutes the excess of bromine was displaced by adding 10 cc. of sodium iodide solution (4 per cent.) and the liberated iodine titrated with sodium thiosulphate solution using starch as indicator. The estimations were generally made after periods of 6 hours and 24 hours, since the amount of material at our disposal did not allow of observations at more frequent intervals. These times were chosen as the preliminary experiments with caseinogen showed that the absorption began after about 1 hour and reached a maximum in from 3–5 hours and that another rise sometimes occurred in about 24 hours. The presence of tryptophane in solution was tested for by the bromine reaction in some experiments; it was generally absent in the 6-hour period but was faintly visible in the 24-hour period.

The estimation with silk-fibroin was performed after hydrolysis with 20 per cent. sulphuric acid for 18 hours as trypsin has only a very slight action upon this protein.   Silk-fibroin does not contain tryptophane.

Peptone Roche, which is prepared from silk-fibroin by acid hydrolysis, gave only a slight precipitate with phosphotungstic acid; it seems to contain tyrosine or a polypeptide containing tyrosine which reacts with bromine. Like silk-fibroin this protein contains no tryptophane.

It was impossible to estimate the tyrosine content of the alcohol soluble proteins—the gliadins—since they are only digested with extreme slowness by trypsin.

The following are the data:

Trypsin.   (Used in experiments with caseinogen, " peptone Roche.")

1 per cent. in 0·25 per cent. Na_2CO_3 solution digested in presence of carbon tetrachloride. 50 cc. samples in 100 cc. phosphotungstic acid solution.   100 cc. filtrate ($=0·33$ g. trypsin) for estimation.   5 cc. sodium bromate solution ($=0·0768$ g. Br).   Titrated with thiosulphate (1 cc. $=0·00672$ g. Br).

Time (hours)	Thiosulphate required	Br absorbed
0	8·3 cc.	0·0210 g.
6	6·3	0·0345
7	6·65	0·0321
24	6·7	0·0318

Caseinogen.   (Hammarsten.)

12·233 g. in 487·27 cc. of 0·25 Na_2CO_3 solution $+122·33$ cc. trypsin solution $+2$ cc. CCl_4. 50 cc. samples in 100 cc. phosphotungstic acid solution. . 100 cc. filtrate ($=0·667$ g. caseinogen $+0·0667$ g. trypsin). 10 cc. sodium bromate solution ($=0·1536$ g. Br).   Titrated with thio- sulphate (1 cc. $=0·00672$ g. Br).

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br absorbed after deducting 0·00642 for trypsin	Tyrosine content per cent.
0	22·9	—	—	—
5 hours	12·5	0·0696	0·0632	5·35
6 ,,	12·65	0·0695	0·0631	5·34
23 ,,	10·75	0·0814	0·0749	6·35
30 ,,	7·4	0·1036	0·0972	8·23
48 ,,	7·9	0·1005	0·0941	7·98
4 days	7·55	0·1036	0·0972	8·23

" *Peptone Roche.*"

5·735 g. in 514·2 cc. of 0·25 Na_2CO_3 solution $+57·3$ cc. trypsin solution $+2$ cc. CCl_4.   50 cc. samples in 100 cc. phosphotungstic acid solution.   100 cc. filtrate ($=0·333$ g. peptone $+0·0333$ g. trypsin) for estimation.   10 cc. sodium bromate ($=0·1536$ g. Br).   Titrated with thiosulphate (1 cc. $=0·00672$ g. Br).

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br absorbed after deducting 0·0021 at 0 hrs. & 0·0032 afterwards for trypsin	Tyrosine content per cent.
0	14·85	0·0998	0·0538	8·79
2·5 hours	14·2	0·0954	0·0582	9·32
3·75 ,,	13·5 ⎫			
7 ,,	13·1 ⎬ 13·4	0·090	0·0636	10·23
9 ,,	13·3 ⎪			
24 ,,	13·6 ⎭			

Silk-fibroin.

4·39 g. were hydrolysed with 20 per cent. H$_2$SO$_4$, and the solution made up to 500 cc. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (=0·293 g. fibroin) for estimation. 5 cc. bromate solution (=0·0768 g. Br). Titrated with thiosulphate (1 cc.=0·00672 g. Br). Thiosulphate required=4·1 cc. Br absorbed=0·0493 g. Tyrosine content=9·53 per cent.

Conglutin (Merck) containing 5·2 per cent. moisture.

5·6158 g. in 222·7 cc. Na$_2$CO$_3$ solution+56·1 cc. trypsin solution+2 cc. CCl$_4$. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (=0·632 g. dry conglutin) for estimation. 5 cc. bromate (=0·0768 g. Br). Titrated with thiosulphate (1 cc.=0·00672 g. Br)

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br. absorbed after deducting 0·00642 for trypsin	Tyrosine content per cent.
0	11·7	—	—	—
4 hours	8·55	0·0193	0·0129	1·15
6 ,,	8·3	0·0210	0·0146	1·31
24 ,,	5·35	0·0415	0·0351	3·15

Excelsin. (Own preparation.)

2·697 g. in 306 cc. of 0·25 per cent. NaOH+2 cc. CHCl$_3$+2 "holadin" capsules. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (=0·2937 g. excelsin) for estimation.

Time	Total Br absorbed, g.	Br absorbed after deducting 0·002 for holadin	Tyrosine content per cent.
0	0·00287	0·0008	
5·5 hours	0·0149	0·0129	2·5
24 ,,	0·0258	0·0238	4·6
28 ,,	0·026	0·024	4·61
48 ,,	0·0282	0·026	5·0

Legumin (Osborne) containing 4·07 per cent. moisture.

2·9866 g. in 266·6 cc. Na$_2$CO$_3$ solution+30 cc. trypsin solution+2 cc. CCl$_4$. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (=0·03198 g. dry legumin+0·0033 g. trypsin) for estimation. 5 cc. bromate (=0·08375 g. Br). Titrated with thiosulphate (1 cc.=0·0079 g. Br).

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br absorbed after deducting 0·0033 g. for trypsin	Tyrosine content per cent.
0	10·45	0·0012	—	—
6·75 hours	8·15	0·0185	0·0152	2·69
27·5 ,,	7·0	0·0272	0·0239	4·23
3 days	6·0	0·0351	0·0318	5·63

Edestin (Osborne) containing 13·07 per cent. moisture.

4·6052 g. in 182·3 cc. Na$_2$CO$_3$ solution+46 cc. trypsin solution+2 cc. CCl$_4$. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (=0·58 g. dry edestin+0·0667 g. trypsin) for estimation. 5 cc. bromate (=0·0768 g. Br). Titrated with thiosulphate (1 cc.=0·00672 g. Br).

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br absorbed after deducting 0·00642 g. for trypsin	Tyrosine content per cent.
0	11·4	—	—	—
4 hours	8·35	0·0207	0·0143	1·4
6 ,,	7·85	0·0241	0·0177	1·73
24 ,,	5·0	0·0432	0·0368	3·6
48 ,,	2·6	0·0593	0·0529	5·17

Vignin (Osborne) containing 7·03 per cent. moisture.

1·8982 g. in 169 cc. Na$_2$CO$_3$ solution + 18·9 cc. trypsin solution + 2 cc. CCl$_4$. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (= 0·310 g. dry vignin + 0·033 g. trypsin) for estimation. 5 cc. bromate (= 0·08375 g. Br). Titrated with thiosulphate (1 cc. = 0·0079 g. Br).

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br absorbed after deducting 0·0033 g. for trypsin	Tyrosine content per cent.
0	10·9	—	—	—
6·75 hours	7·95	0·0223	0·0190	3·4
27·5 ,,	6·35	0·0360	0·0327	5·97

Squash Seed Globulin (Osborne) containing 10·02 per cent. moisture.

1·912 g. in 170·1 cc. Na$_2$CO$_3$ solution + 19·1 cc. trypsin solution + 2 cc. CCl$_4$. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (= 0·3 g. dry protein + 0·0333 g. trypsin) for estimation. 5 cc. bromate (= 0·08375 g. Br). Thiosulphate (1 c.c. = 0·0079 g. Br).

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br absorbed after deducting 0·0033 g. for trypsin	Tyrosine content per cent.
0	10·8	—	—	—
5·75 hours	8·2	0·0205	0·0172	3·24
27 ,,	6·5	0·0339	0·0306	5·8

Amandin (Osborne) containing 10·46 per cent. moisture.

3·3404 g. in 298·6 cc. Na$_2$CO$_3$ solution + 33·4 cc. trypsin solution + 2 cc. CCl$_4$. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (0·3 g. dry amandin + 0·0333 g. trypsin) for estimation. 5 cc. bromate (= 0·08375 g. Br). Thiosulphate (1 cc. = 0·0079 g. Br).

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br absorbed after deducting 0·0033 g. for trypsin	Tyrosine content per cent.
0	10·6	—	—	—
6 hours	8·2	0·01896	0·0157	2·9
24 ,,	6·75	0·0304	0·0271	5·1

Glycinin (Osborne) containing 9·06 per cent. moisture.

1·9166 g. in 170·5 cc. Na$_2$CO$_3$ solution + 19·2 cc. trypsin solution + 2 cc. CCl$_4$. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (= 0·302 g. dry glycinin + 0·0333 g. trypsin) for estimation. 5 cc. bromate (= 0·08375 g. Br). Thiosulphate (1 cc. = 0·0079 g. Br).

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br absorbed after deducting 0·0033 g. for trypsin	Tyrosine content per cent.
0	10·4	—	—	—
6 hours	9·3	0·0091	0·00058	1·1
28 ,,	7·7	0·0218	0·0185	3·47

In most cases there was an increase in the bromine absorption between the periods of 6 hours and 24 hours. The exact time at which the increase occurred could not be ascertained since it was impossible to make more frequent determinations owing to the scarcity of material. An increase after a constant period would show the point when all the tyrosine was liberated and that at which the tryptophane became set free. There is a very close

agreement between the figures for the tyrosine content after the 6-hour interval and the figures obtained by isolation and weighing, as is shown by the following table :

Protein	Percentage of tyrosine after 6 hours' digestion	Percentage by weighing
Caseinogen	5·34	4·5
" Peptone Roche"	10·23	—
Silk-fibroin	9·53	9–10·5
Conglutin	1·31	2·1
Legumin	2·69	2·4
Edestin	1·73	2·1
Vignin	3·4	2·3
Squash seed globulin	3·24	3·1
Amandin	2·9	1·1
Glycinin	1·1	1·9
Excelsin	2·5	3·1

The correspondence in the figures in the cases of edestin, glycinin, squash seed globulin, excelsin, legumin and silk-fibroin is very close. The value is 1 per cent. higher for caseinogen, and the value is also higher for amandin and vignin. The result should be slightly higher for excelsin as the material was not dried. The amounts of amandin and glycinin available were very small, so that much stress cannot be placed on these figures.

The method of bromination therefore appears to be of use for the estimation of the tyrosine content of proteins if measurements of the absorption are made at frequent intervals during a tryptic digest of the protein, but it must be used with precautions and the figures carefully criticised.

SUMMARY.

The estimation of small quantities of tyrosine—0·01–0·04 g.—can be effected by J. H. Millar's method of bromination, when a more dilute solution of sodium bromate is used, but it is preferable to modify his procedure by adding excess of the reagent and titrating the non-absorbed halogen with thiosulphate solution, using potassium iodide and starch as indicator. Tyrosine cannot be directly estimated by bromination in the presence of protein and its decomposition products, since histidine and tryptophane also absorb bromine. Histidine can be removed by precipitation with phosphotungstic acid. The absorption of bromine by tryptophane is not completely eliminated after boiling with acids so that tyrosine cannot be estimated by this method in solutions containing the products of acid hydrolysis of proteins which contain tryptophane. Values for the tyrosine

content of proteins, agreeing with those obtained by isolation and weighing, are obtained when the bromine absorption of a tryptic digest is measured after an interval of about 6 hours.

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REFERENCES.

Abderhalden and Fuchs (1913), *Zeitsch. physiol. Chem.* **83,** 468.
Auld and Mosscrop (1913), *J. Chem. Soc.* **103,** 281.
Brown, A. J. and Millar, E. T. (1906), *J. Chem. Soc.* **89,** 145.
Folin and Denis (1912), *J. Biol. Chem.* **12,** 245.
Hopkins and Cole (1901), *J. Physiol.* **27,** 418.
Knoop (1908), *Beiträge*, **11,** 356.
Millar, J. H. (1903), *Trans. Guinness Research Laboratory*, **1,** Part 1, 40.
Osborne and Clapp (1906), *Amer. J. Physiol.* **17,** 246.
—— and Guest (1911), *J. Biol. Chem.* **9,** 388.
Plimmer (1913), *Biochem. J.* **7,** 311.

XXXI. THE SEPARATION OF CYSTINE AND TYROSINE.

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Cystine and tyrosine resemble each other so closely in their solubility in water, in alkali and in acid, that their separation is not easily effected. Both Mörner [1899, 1901] and Embden [1900] who first isolated cystine from the products of the acid hydrolysis of scleroproteins obtained it mixed with tyrosine. Mörner effected the separation of the two substances by fractional crystallisation from ammonia; Embden by dissolving out the tyrosine with very dilute nitric acid. Friedmann [1902] separated tyrosine from cystine by solution in ammonia and neutralisation with acetic acid; tyrosine crystallised out in the neutral solution and the cystine was precipitated by making the filtrate strongly acid with acetic acid. Cystine is usually prepared from the products of acid hydrolysis of proteins by nearly neutralising with soda and allowing to crystallise. Folin [1910] described the isolation of cystine and tyrosine by neutralising the hydrochloric acid hydrolysis solution to Congo-red with sodium acetate; cystine crystallises out and on diluting the filtrate and allowing it to stand the tyrosine separates out slowly. Cystine thus seems to be the more insoluble in dilute mineral acids and in strong acetic acid.

Neither Mörner's, Embden's, nor Friedmann's method gives a quantitative separation of the two substances; they only permit of the isolation of a portion of the mixture. Folin's method of preparation was devised for obtaining a quantity of cystine. A method for the separation of these two constituents of a protein is therefore required. On account of their similarity the tyrosine isolated from the products of hydrolysis may contain cystine unless this unit has been completely decomposed during the process of

hydrolysis[1]. For this reason the data of the actual amount of cystine in most proteins are scanty or lacking and except in the scleroproteins and in the protein described by Kotake and Knoop [1911] the relative amount of cystine to tyrosine is very small. The estimations of tyrosine which have been made by Plimmer and Miss Eaves [1913] by the method of bromination are not greatly higher than the amounts of tyrosine obtained by direct isolation and weighing. The presence of a small amount of cystine in the tyrosine may explain the slight differences.

Winterstein [1901] described the precipitation of cystine by phosphotungstic acid and Hopkins and Cole [1902] its precipitation by a solution of mercuric sulphate in five per cent. sulphuric acid. Tyrosine is not precipitated by phosphotungstic acid and its mercury compound is soluble in the five per cent. sulphuric acid. Cystine and tyrosine have been found to differ very greatly in their behaviour to absolute alcohol saturated with hydrochloric acid gas. Tyrosine is readily esterified and goes into solution in the acid alcohol; cystine is not readily esterified and is only very slowly dissolved and the portion which goes into solution can be precipitated by adding an equal volume of absolute alcohol. Neither of these differences has hitherto been used for the specific purpose of separating the two compounds and their applicability has therefore been tested.

EXPERIMENTAL.

Tyrosine was prepared by the hydrolysis of caseinogen by trypsin. The products which crystallised out during the digestion and on the concentration of the filtered solution were mixed and purified by repeatedly dissolving in dilute sulphuric acid and exactly neutralising with caustic soda, 14 g. of pure tyrosine being thus obtained from 600 g. of caseinogen. The substance gave no precipitate with phosphotungstic acid showing the absence of diamino-acid (diaminotrihydroxydodecanic acid) and of cystine.

Cystine was prepared from wool and hair by Folin's method; the final product was not perfectly white but it consisted of the typical hexagonal plates.

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[1] Cystine is rapidly decomposed by boiling with alkali with the formation of hydrogen sulphide (loosely bound sulphur) and it is also decomposed by prolonged boiling with acid. In preparing cystine from wool or hair the best yield was obtained when the material was boiled with concentrated hydrochloric acid for 3–4 hours; the yield was very poor when the boiling lasted from 5–8 hours. In purifying the cystine by boiling with charcoal in acid solution loss also occurs. If a solution of cystine in dilute hydrochloric acid be boiled for a long time it becomes yellow or yellow-brown in colour.

(1) *Separation by means of Phosphotungstic Acid.*

Cystine and tyrosine were mixed together in different proportions and dissolved in 50 cc. of 5 per cent. sulphuric acid. A 30 per cent. solution of phosphotungstic acid in 5 per cent. sulphuric acid was added so long as a precipitate was formed. (20 cc. sufficed for the precipitation of 0·5 g. of cystine.) After standing for 12 hours the precipitate was filtered off and washed repeatedly with a 2·5 per cent. solution of phosphotungstic acid in 5 per cent. sulphuric acid.

The cystine was recovered from the precipitate either by suspending it in water or in water containing acetone as recommended by Wechsler [1911] and adding baryta water until the solution remained permanently alkaline to phenolphthalein, the decomposition being carried out on the water bath. Excess of baryta was carefully avoided so as to prevent decomposition of the cystine. The filtrate from the barium phosphotungstate was acidified with hydrochloric acid, evaporated to a small volume on the water bath, and neutralised with ammonia. The cystine crystallised out and after being left to stand for 1–2 days was filtered off, washed, dried and weighed.

The recovery of the cystine from its phosphotungstate by decomposition with hydrochloric acid and extraction of the reagent with ether (Winterstein) was also attempted; the whole of the reagent was not dissolved by the ether and the recovered cystine was contaminated with phosphotungstic acid. Experiments were not made with amyl alcohol which Jacobs [1912] recommended as a solvent for extracting phosphotungstic acid.

The tyrosine was recovered from the filtrate by adding ammonia to remove the excess of phosphotungstic acid, filtering off the ammonium phosphotungstate, neutralising and evaporating to a small volume. The crystals so obtained were washed with water to remove ammonium sulphate and the tyrosine residue was dried and weighed. This procedure was preferred to the usual method of removing excess of acid with baryta which entails repeated extraction of a bulky precipitate of barium phosphotungstate and sulphate with hot water and the evaporation of a large volume of liquid.

The amounts of cystine and tyrosine taken and recovered were as follows:

Taken		Recovered	
Cystine	Tyrosine	Cystine	Tyrosine
0·5 g.	0·5 g.	0·35 g.	0·35 g.
0·5	1·0	0·47	0·6
0·5	1·0	0·45	0·55
1·0	0·5	0·80	0·25
1·0	0·5	0·82	0·4
0·5	—	0·27	—
0·5	—	0·3	—

Neither the cystine nor the tyrosine was completely recovered. The cystine is precipitated practically completely by the phosphotungstic acid; the loss seems to take place in its recovery from the phosphotungstic acid precipitate; the solution must be made slightly alkaline to ensure complete decomposition of the phosphotungstate and some of the cystine is most probably also decomposed by the alkali. Some decomposition may also occur during the evaporation of the acid solution. Except in the last experiment the cystine always contained tyrosine and this accounts for the loss of tyrosine. The cystine was only obtained free from tyrosine in the last experiment in which the precipitate was washed some twenty times by removing it from the filter, stirring up with the washing reagent and again filtering until the washings showed no reaction with Millon's reagent: over 80 per cent. of the tyrosine was then recovered.

2 g. of the cystine recovered from the earlier experiments were found to contain 0·6 g. of tyrosine which was isolated by means of alcohol saturated with hydrochloric acid (as described below).

(2) *Separation by means of Mercuric Sulphate.*

Mixtures of cystine and tyrosine in various proportions were made and dissolved in 5 per cent. sulphuric acid and treated with mercuric sulphate dissolved in 5 per cent. sulphuric acid (Hopkins and Cole's tryptophane reagent) until no further precipitate occurred. The precipitate was filtered off after standing for 12 hours and washed repeatedly with 5 per cent. sulphuric acid by removing from the filter, stirring up with the acid and again filtering until the washings gave no reaction with Millon's reagent.

The cystine was recovered from the precipitate by suspending in water and decomposing with hydrogen sulphide. The filtrate from the mercuric sulphide was evaporated on the water bath to a small volume and then neutralised with ammonia. The cystine crystallised out and was filtered off, washed, dried and weighed. The acid filtrate containing the tyrosine was evaporated on the water bath to about 400 cc. and filtered from the mercuric sulphate which had separated out. A slight excess of ammonia was added and after again filtering the solution was evaporated almost to dryness. The crystals so obtained were filtered off and washed with water until free from ammonium sulphate and the residue of tyrosine was dried and weighed. This procedure was preferred to the removal of the sulphuric acid with baryta which would have necessitated the repeated extraction of the insoluble barium sulphate with boiling water.

As with the previous method the amounts of cystine and tyrosine recovered were far from quantitative as is shown by the following figures:

Taken		Recovered	
Cystine	Tyrosine	Cystine	Tyrosine
0·5 g.	—	0·30 g.	—
0·5	—	0·31	—
0·5	0·5 g.	0·26	0·45 g.
0·5	0·5	0·28	0·47
0·5	1·0	0·31	0·75
0·5	1·0	0·15	0·96

The loss of tyrosine was apparently less than that of cystine but the tyrosine was very impure and contained a brown pigment arising from the decomposition of cystine. On further investigation the precipitation of cystine by mercuric sulphate in 5 per cent. sulphuric acid was found to be incomplete, as was shown by an estimation of the nitrogen in an experiment with cystine alone:

1 g. of cystine was dissolved in 100 cc. of 5 per cent. sulphuric acid; 20 cc. were found to contain 0·0224 g. N by Kjeldahl's method. The remaining 80 cc. (= 0·0896 g. N) were precipitated with 21 cc. mercuric sulphate solution. 70 cc. of the filtrate contained 0·0172 g. N.

Hence the amount precipitated was 0·0724 g. or 81 per cent. of the cystine. The amount of cystine recovered from the precipitate by the procedure described above was 0·3 g. instead of 0·6 g. Loss occurs not only in the precipitation but also in evaporating the solution before neutralising with ammonia. Cystine is much more unstable to acid than one is led to expect from the description of its isolation.

(3) *Absolute alcohol saturated with hydrogen chloride.*

Whilst preparing tyrosine ethyl ester from some tyrosine it was observed that complete solution of the material could not be effected and an examination of the insoluble residue showed it to be cystine; 5 g. of the material yielded 0·05 g. cystine and 4 g. yielded 0·2 g. cystine

This difference in the behaviour of cystine and tyrosine suggested a simple method for effecting their separation.

Preliminary experiments were made with pure cystine and pure tyrosine; 0·5 g. cystine was covered with 20 cc. absolute alcohol saturated with hydrogen chloride, warmed on the water bath and allowed to stand for 12 hours. The undissolved substance was filtered off, washed with absolute alcohol, dried and weighed. Yield = 0·39 g. A white precipitate was produced when the wash alcohol came into contact with the filtrate. This

precipitate was filtered off, washed, dried and weighed. Yield = 0·11 g. On dissolving a test portion of each of these quantities in ammonia and allowing to crystallise the typical hexagonal plates characteristic of cystine were formed.

0·5 g. tyrosine was covered with absolute alcohol saturated with hydrogen chloride and warmed on the water bath. Complete solution readily occurred and the tyrosine was converted into its ethyl ester. On adding water and neutralising with sodium carbonate no tyrosine was precipitated, but on acidifying and boiling for 4–5 hours the ester was hydrolysed and on again neutralising with sodium carbonate tyrosine was precipitated. It was filtered off, washed, dried and weighed. Yield = 0·45 g. A further 0·05 g. was obtained on acidifying the filtrate, boiling, and neutralising once more.

A mixture of 0·5 g. of each was then treated in the same way with 30 cc. absolute alcohol saturated with hydrogen chloride. 0·35 g. was insoluble and the alcohol used for washing the residue precipitated an additional 0·1 g. The filtrate after hydrolysis of the tyrosine ester yielded 0·47 g. tyrosine.

In these experiments the cystine was recovered in two fractions, but if an equal volume of absolute alcohol be added to the absolute alcohol saturated with hydrogen chloride before filtering the whole of the cystine can be obtained in one operation. The cystine which goes into solution seems to be cystine hydrochloride, for it dissolves in water. The insoluble portion seems to consist mainly of cystine but a portion of it dissolves if it be washed with water, and is apparently cystine hydrochloride as the cystine is precipitated in hexagonal plates on neutralising with ammonia. There is no evidence that the cystine is converted into its ester. According to Friedmann cystine is not easily converted into its ethyl ester but its methyl ester is more easily obtained (Fischer and Suzuki).

The separation of cystine and tyrosine by this method has been tested by the following experiments:

Taken		Recovered	
Cystine	Tyrosine	Cystine	Tyrosine
0·5 g.	0·0 g.	0·47 g.	0·0 g.
0·5	0·0	0·50	0·0
0·0	0·5	0·0	0·5
0·0	0·5	0·0	0·49
0·5	0·5	0·45	0·47
0·5	1·0	0·49	0·96
1·0	0·5	1·00	0·50

The mixtures were treated with absolute alcohol saturated with hydrogen chloride and warmed on the water bath. An equal volume of alcohol was added and the insoluble cystine filtered off, washed, dried and weighed. The

filtrate was diluted with 2 volumes of water and boiled for 8 hours, water being added when necessary. Tyrosine was precipitated on neutralising; it was filtered off, washed, dried and weighed.

The usefulness of this method is illustrated by the first experiment in which presumably pure tyrosine prepared from wool by Folin's method had been used. The presence of cystine was not observed by microscopic examination and the cystine present was found to be unevenly distributed.

The cystine recovered from the phosphotungstate precipitate above mentioned contained tyrosine; 2 g. contained 1·4 g. cystine and 0·6 g. tyrosine.

A mixture weighing 3 g. was found to contain 1·9 g. cystine; the tyrosine was unfortunately lost. This mixture actually contained 2 g. cystine and 1 g. tyrosine.

SUMMARY.

1. Cystine and tyrosine can be separated from one another by precipitation with phosphotungstic acid. The precipitation of cystine is almost complete, but loss occurs in its recovery from the precipitate. Almost the whole of the tyrosine can be recovered from the filtrate and washings.

2. Cystine and tyrosine can be separated from one another by precipitation with mercuric sulphate in five per cent. sulphuric acid. The cystine is not completely precipitated and the tyrosine which is recovered is impure.

3. Cystine and tyrosine can be completely and quantitatively separated by means of absolute alcohol saturated with hydrogen chloride. The tyrosine is rapidly converted into tyrosine ester and goes into solution. It can be recovered by boiling the solution when diluted with water for eight hours and then neutralising with ammonia. Almost the whole of the cystine is insoluble; the portion which goes into solution (perhaps cystine hydrochloride) is precipitated by adding an equal volume of absolute alcohol. The cystine is not converted into its ethyl ester since on dissolving the insoluble portion in dilute hydrochloric acid and neutralising with ammonia the cystine is precipitated in the typical hexagonal plates.

REFERENCES.

Embden (1900), Zeitsch. physiol. Chem. 32, 94.
Folin (1910), J. Biol. Chem. 8, 9.
Friedmann (1902), Beiträge, 3, 1.
Hopkins and Cole (1902), J. Physiol. 27, 418.
Jacobs (1912), J. Biol. Chem. 12, 429.
Kotake and Knoop (1911), Zeitsch. physiol. Chem. 75, 488.
Mörner (1899), Zeitsch. physiol. Chem. 28, 595.
—— (1901), Zeitsch. physiol. Chem. 34, 207.
Plimmer and Eaves (1913), Biochem. J. 7, 297.
Wechsler (1911), Zeitsch. physiol. Chem. 73, 138.
Winterstein (1901), Zeitsch. physiol. Chem. 34, 153.

XXXII. THE FACTORS CONCERNED IN THE SOLUTION AND PRECIPITATION OF EUGLOBULIN.

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CONTENTS.

I. INTRODUCTION.

There is no reason for regarding euglobulin, the material precipitated from serum by dialysis or by dilution and acidification, as a chemical entity. Michaelis and Rona [1910, 2] are of opinion that it consists of that portion of the total proteins which owes its solution to the dispersing power of the electric charge upon its particles. It is true that the procedure adopted for separating euglobulin is one which renders it iso-electric with the solution; but the conditions determining its dispersion in serum are not so simple as they suggest. Euglobulin, as shown by Hardy [1905], is dissolved by neutral salts, e.g., sodium chloride, in a concentration of 1/10th normal, to form a colloidal solution in which the particles are electrically neutral, a result which I have been able to confirm (see p. 329, below). Serum contains enough salt to produce this effect and unless it is at the same time diluted, the euglobulin it contains cannot be completely precipitated by the addition of the amount of acid necessary to render it iso-electric. The dispersion of euglobulin in serum is due to two entirely distinct causes: (1) Electric charge on the particles owing to the alkalinity of the fluid; (2) Formation of a "soluble" compound with the salt present,

concerning the mechanism of which there are a variety of theories, which are discussed in detail below.

By the process of "heat-denaturation" proteins acquire many of the properties peculiar to euglobulin, as regards the factors conditioning their solution (dispersion) and agglutination (see Hardy [1900], Michaelis and Mostynski [1910] and Chick and Martin [1912]). The results of the last-named, however, afford no support for the opinion expressed by Starke [1900] and Moll [1904] that "albumin" is converted into "globulin" by heating. Abderhalden [1903, 1904] has found evidence of difference in chemical composition between the two sets of proteins which is not obliterated after the former has been heated. Further, the analogy mentioned above is by no means complete; the union of euglobulin with neutral salts to form a dispersion that is without drift in an electric field has no parallel in the case of denaturated serum-proteins.

II. Solution of Euglobulin by Acids and Alkalies, and the Iso-Electric point.

Solution of euglobulin by means of acid or alkali was shown by Hardy [1905] to be associated with the possession by the dispersed particles of an electric charge which was respectively positive or negative in sign.

Michaelis and Rona (1910, 2) found the iso-electric point of euglobulin to be at a concentration of hydrogen-ions equal to 36×10^{-7} normal and to coincide with the point of optimum flocculation for this protein.

The iso-electric point has been re-determined in the present instance and the result of Michaelis and Rona has been confirmed.

The euglobulin was prepared as follows: horse serum was diluted ten times with distilled water and the globulin was precipitated by acidifying with acetic acid (about 3–4 cc. N acetic acid per litre according to the original reaction of the serum). The precipitate was allowed to settle, was centrifuged off, and purified by dissolving in a minimal amount of standard sodium hydroxide solution (according to the amount of the precipitate) and reprecipitating with hydrochloric acid, the precipitate again being separated by centrifuging. This operation was repeated once or twice and the precipitate finally washed with distilled water. A fairly concentrated suspension was made in distilled water from which, by dilution, the material was prepared which was used for the various experiments[1]. The particles of

[1] In some experiments, for example those in Tables IV and V, the euglobulin was prepared from horse-plasma (oxalate) by the method described by Mellanby [p. 339, 1905]. The purification in this case was just as above and no differences were detected between samples prepared by the two methods.

suspensions prepared as above invariably had a slight negative charge and the addition of a little acid was necessary in order to render the particles iso-electric with the solution.

In Table I is shown the degree of dispersion of a sample of euglobulin (0·032 % solution) corresponding with various concentrations of acid and alkali. The charge carried by the particles was at the same time determined

TABLE I.

Influence of reaction (hydrogen-ion concentration) upon the dispersion of euglobulin, and the electric charge carried by the protein particles; influence of sodium sulphate.

Concentration of protein = 0·032 %.

Exp. No.	Cc. N/100 HCl (or equivalent) added in total volume of 10 cc.	Cc. N/100 NaOH (or equivalent) added in total volume of 10 cc.	Salt added	Concentration of salt, in terms of normality	Concentration of hydrogen-ions, in terms of normality	Sign of electric charge on the particles	Degree of agglutination
1	—	0·2	—	—	$0·36 \times 10^{-7}$	–	Faintly opalescent soln.
2	—	0·1	—	—	0·97 ,,	–	Opalescent solution.
3	—	0·0	—	—	32 ,,	–	Agglutinated.
4	·1	—	—	—	748 ,,	–	Agglutinated later.
5	·15	—	—	—	1140 ,,	+	Opalescent solution.
6	·2	—	—	—	1390 ,,	+	Faintly opalescent soln.
7	·5	—	—	—	5080 ,,		Clear solution.
8	·2	—	Na_2SO_4	0·03	0·97 ,,	–	Opalescent solution[1].
9	·5	—	,,	0·05	1590 ,,	–	Opalescent solution[1].

[1] Agglutinated on standing.

by observing their behaviour in an electrical field, using the microscopic method previously employed by Martin and the author [1912, p. 285] in investigating the electrical properties of denaturated proteins. In the 5th column is given the concentration of hydrogen-ions in the various solutions, and the point of optimum agglutination is seen to be at a concentration equal to 32×10^{-7} normal. This figure is in good agreement with that found by Michaelis and Rona.

Confirmation of these values was incidentally obtained in the course of experiments made to elucidate other points. For example, the range of agglutination of another euglobulin suspension, containing 0·6 % protein was determined, after first dissolving in a minimum amount of dilute sodium hydroxide solution, by adding dilute hydrochloric acid to a series of tubes until precipitation occurred and finally dispersion was again obtained. Dispersion corresponded with a concentration of hydrogen-ions equal to $2·2 \times 10^{-7}$

normal on the alkaline side and $62 \cdot 1 \times 10^{-7}$ normal on the acid side. The solutions, of course, all contained a trace of salt.

In case of a third sample of euglobulin, see Table II (containing $0 \cdot 016 \, {}^0/_0$ protein) the limits of agglutination were found to lie between concentrations of hydrogen-ions equal to 5×10^{-7} and 213×10^{-7} normal and precipitation to be rapid at a concentration of 18×10^{-7} normal.

TABLE II.

Influence of reaction (hydrogen-ion concentration) upon the dispersion of euglobulin and upon the electric charge carried by the protein particles; influence of sodium sulphate.

Concentration of protein $= 0 \cdot 016 \, {}^0/_0$.

Exp. No.	Salt added	Concentration of salt added, in terms of normality	Cc. N/100 HCl (or equivalent) added in total volume of 10 cc.	Cc. N/100 NaOH (or equivalent) added in total volume of 10 cc.	Concentration of hydrogen-ions, in terms of normality	Sign of electric charge on particles	Degree of agglutination
1	—	—	0·3	—	—	+	Clear solution.
2	—	—·	0·2	—	1280×10^{-7}	+	,, ,,
3	—	—	0·1	—	213 ,,	+	Agglutination partial.
4	—	—	0·00	—	18·6 ,,	—	Agglutination complete.
5	—	—	—	0·1	5·41 ,,	—	Agglutination partial.
6	—	—	—	0·2	—	—	Dispersed.
7	Na$_2$SO$_4$	·02	0·5	—	1010×10^{-7}	—	Agglutination partial, less good than No. 8.
8	,,	·03	,,	—	137 ,,	—	Agglutination best, but not quite complete.
9	,,	·04	,,	—	65·9 ,,	—	Agglutination less good than No. 8.
10	,,	·05	,,	—	—	0	Dispersed.
11	,,	·07	,,	—	—	0	Dispersed.

The experiments as to the amount and sign of the charge carried by the euglobulin particles, detailed in Tables I and II, are not calculated to define the iso-electric point with as great accuracy as those of Michaelis and Rona. As a rule the particles, while agglutinating rapidly, retained the negative charge which they originally held and only showed a positive charge when dispersion by acid was already well begun. Speaking generally, however, the point where the charge on the particles changed its sign coincided with the agglutination-zone. On the whole, there is a tendency to retain the negative charge rather than the positive. A distinctly positive charge was first detected on the globulin particles at a concentration of hydrogen-ions

equal to about 200×10^{-7} normal (see Exp. 3, Table II, and Exp. 2, Table VII).

The agglutination and dispersion of euglobulin by acids and alkalies, in confirmation of the results of Michaelis and Rona, is seen to be primarily dependent upon hydrogen-ion concentration, and in this respect a close analogy is presented with heat-denaturated proteins [Michaelis and Rona, 1910, 1; Sörensen and Jürgensen, 1911; Chick and Martin, 1912]. In both cases flocculation takes place when the protein particles are iso-electric with the solution, and, in cases where the reaction of the solution is more acid or alkaline than the iso-electric point, dispersion of the protein is due to the possession of a positive and negative electric charge respectively.

III. Solution of Euglobulin by Neutral Salts.

There has been some difference of opinion as to the mechanism involved in the solution of euglobulin by electrolytes. Hardy [1905] came to the conclusion that salt-solutions of globulin are without drift in an electric field and the particles of the system must be regarded as electrically neutral. He considered [1905, p. 325] "salt-globulin" to be the result of a molecular union of globulin and salt[1] by a process analogous to the formation of amino-acid-salt compounds[2], thus:

$$
\begin{array}{ccc}
& & \text{Na}\quad\text{Cl} \\
& & \diagdown\diagup \\
\text{N}=\text{H}_2 & & \text{N}=\text{H}_2 \\
\diagup & & \diagup \\
\text{R} \qquad +\text{NaCl}= & \text{R} \\
\diagdown\;\;\diagup\text{O} & & \diagdown\;\;\diagup\text{O} \\
\text{C} & & \text{C} \\
\diagdown\text{OH} & & \diagdown\text{OH}
\end{array}
$$
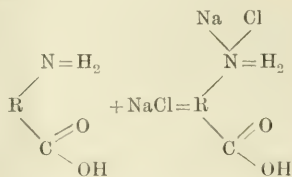
A similar theory had already been put forward by Pauli [1899].

Mellanby [1905], on the other hand, has expressed the opinion that the solution of globulin by electrolytes is the result of activity of the constituent ions. He found that the efficiency of a salt in "dissolving" euglobulin depended on the valency of its ions, and, moreover, that the amount of globulin dissolved by a given concentration of any salt was proportional to the initial concentration of the protein. This curious fact was also noticed by Hardy [1905, p. 310].

A third view is that of Schryver [1910]. He considers (1) that, when precipitated, euglobulin is in a dehydrated condition, the amphoteric molecules having united by twos in salt formation with loss of the elements of

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[1] Osborne and Harris [1905] also interpreted the solution of edestin by certain electrolytes as due to the formation of such compounds.

[2] These appear to have been prepared in the crystalline form by Pfeiffer and Modelski [1912].

water and (2) that, when dissolved by electrolytes, the molecules remain separate in their hydrated condition, the aggregation in pairs being prevented by adsorption of the electrolytes on the surface of the molecules, thus

$$
\begin{array}{ccc}
\underset{\text{R}}{\overset{\text{COOH \quad NH}_3\text{OH}}{\diagup\,\diagdown}} + \underset{\text{R}}{\overset{}{\diagup\,\diagdown}} & = & \text{H}_2\text{O} + \underset{\text{R}}{\overset{\text{COO}\text{---}\text{NH}_3}{\diagup\,\diagdown}}\underset{}{\overset{}{}}\text{R} \\
\text{NH}_3\text{OH \quad COOH} & & \text{NH}_3\text{OH \quad COOH}
\end{array}
$$

Salt-dissolved globulin (hydrated)   Precipitated (dehydrated) globulin
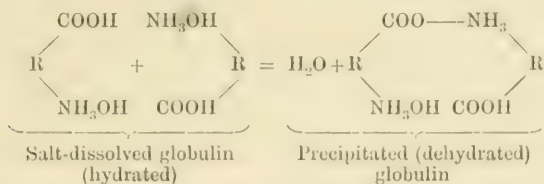
The insolubility of the dehydrated euglobulin Schryver attributes to the increased size of the molecule[1]. There seems to be, however, better experimental evidence in support of the more usual conception that the euglobulin is precipitated in such circumstances as will allow of the aggregation of the protein particles under the influence of surface tension, that is to say, in the absence of other causes tending to keep them dispersed. Such causes are the presence of acid or alkali, in which case the euglobulin particles have been shown to be charged electrically, or the presence of electrolytes in whatever way the latter may act.

Schryver's view is in accord with that of Hardy in so far as the solution of euglobulin by electrolytes is attributed by both to the agency of the salt as a whole rather than to the activity of the constituent ions. His adsorption hypothesis, however, affords no explanation of the comparatively high concentration necessary for solution in case of many salts.

An additional theory, for which some experimental support is here given, is that dispersion of euglobulin by salts may be due to a specific adsorption by the protein particles of one—the more potent—ion of the electrolyte employed, and the acquisition of an electric charge by this means. Such an hypothesis would explain the influence of valency, demonstrated by Mellanby, and be consistent with what is known of the action of neutral salts in dispersing denaturated serum proteins [Chick and Martin, 1912, p. 281], with which euglobulin is in many ways analogous.

In the experiments detailed below, the "solution" of euglobulin by neutral salts was studied, using a series of electrolytes of more varied character, as regards the valency of the constituent ions, than those employed by the above observers. By this means some facts have been discovered which go to show that while, in some instances, solution of euglobulin by

[1] The conclusion of Dabrowski [1912] that in solutions of crystalline egg-albumin containing ammonium sulphate, the colloidal particles are of smaller size than in the case of the dialysed protein, is of interest in this connection.

neutral salts appears to be due to some action of the whole salt, in other cases it is undoubtedly associated with an acquirement of electric charge by the protein particles, the sign of which is determined by the more potent ion of the electrolyte employed.

1. *Experiments to determine whether salt-globulin is electrically charged.*

A series of experiments with a dilute euglobulin suspension (0·016 %protein) and a variety of salts is set forth in Table III. The object was to compare quantitatively the solvent and dispersing power of different ions and to decide whether solution was accompanied by the deposition of an electric charge upon the protein particles. The latter, in this instance, was determined by the microscopic method, using a dark ground and side illumination. The fluids were placed in a specially constructed cell and the behaviour of the particles in an electric field was observed. In using this method with fairly concentrated salt solutions (0·01 normal and upwards) there is some risk of disturbance by gas-bubbles given off at the electrodes, as the result of electrolysis. This has to be very carefully watched. As a rule the difficulty is minimised if the electrodes are previously platinised and the circuit closed only for the short time necessary to make the observation. The electromotive force employed was about 9 volts.

The results of this series of experiments show the relative solvent power of six different salts, viz. the chloride, sulphate and citrate of sodium, the chlorides of barium and calcium and lanthanum nitrate.

It is very clear that the influence of valency in preventing agglutination of euglobulin is far from negligible, multivalent ions being much the more powerful in order of increasing valency. For example, the concentration of lanthanum nitrate and sodium citrate required for the purpose is respectively 1/30th and 1/60th of the necessary concentration of sodium chloride (5th column, Table III).

The electrical condition of the dispersed particles is set out in column 4, and it will be seen that, in four cases, a charge was demonstrated of similar sign to that carried by the more potent ion of the electrolyte employed. In the case of calcium[1] and barium salts, on the other hand, no charge could be detected on the protein particles which remained visible.

The *character* of the dispersion obtained was also different in the case of different salts, and could be grouped according to two types, which I have tentatively called the "electrical" and the "molecular" type of solution.

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[1] This was also found to be the case when denaturated serum-proteins were dispersed by calcium salts [Chick and Martin, 1912, Table XIV].

TABLE III.

Solution of euglobulin by electrolytes.

Concentration of protein $= 0.016$ $^0/_0$.

× × × Complete agglutination, filtrate protein-free.
× ×   Less complete agglutination, filtrate contained trace protein.
×   Partial agglutination.
× −   Almost complete dispersion.
−   Complete dispersion, clear solution.

Salt added	Concentration of salt, in terms of normality	Degree of agglutination	Sign of the electric charge upon the particles	Concentration of salt, in terms of normality, necessary	
				(a) to prevent agglutination	(b) to complete dispersion
NaCl	0·01	× × ×		0·03	—
	0·02	× × ×			.
	0·03	× ×			
	0·04	× ×			
	0·045	× ×	− *		
	0·05	×	− *		
Na₂SO₄	0·0005	× × ×		0·02	0·05
	0·001	× × ×			
	0·01	× × ×			
	0·02	× ×	− *		
	0·03	×			
	0·04	× −	−· *		
	0·05	−			
Na₃Cit	0·0005	×	··	0·0005	0·003
	0·001	×			
	0·002	× −			
	0·003	−			
CaCl₂	0·01	× × ×		0·02	—
	0·02	× ×			
	0·03	× ×			
	0·04	× ×			
	0·05	×	0		
BaCl₂	0·001	× × ×		· 0·005	0·05
	0·005	× ×			
	0·008	× ×			
	0·01	× ×	0		
	0·02	×	0		
	0·03	× −			
	0·05	−	0		
La(NO₃)₃	0·001	× ×	+	0·001	0·02
	0·003	×			
	0·005	× ×			
	0·006	× −	+		
	0·008	× ·			
	0·01	× −			
	0·02	−			

* Agglutinated particles.

That with a lanthanum salt or a citrate was exactly analogous to dispersion by acids and alkalies, the globulin suspension, with progressive increase in concentration of the salt, passed through various gradings of opalescent solutions until a clear transparent fluid was finally obtained. At the same time, under the microscope, using a dark ground illumination, innumerable particles could be seen which became smaller and smaller, acquiring at the same time an unmistakably positive or negative charge. Finally the whole field became a uniform grey in colour, until at length no particles could be distinguished.

In the case of the other salts used, there was usually no grading of opalescence, but, as the concentration of salt was increased, a decreasing amount of the original suspension remained undissolved in an otherwise clear fluid. Under the microscope there was a black and white effect, fewer and fewer agglutinated masses remaining visible on the black ground. This type of solution obtained with sodium sulphate and the chlorides of sodium, calcium and barium. In the case of the two latter salts, no charge was discovered on the visible particles; with sodium chloride and sulphate a negative charge was observed on the agglutinated particles which remained visible. It must however be remembered that the sign of the charge in both cases was negative, i.e. similar to that carried by the original globulin suspension.

The microscopic method is clearly unsuitable for studying the electrical condition of salt-globulin in such cases as the above, where "solution" is associated with a degree of dispersion which renders the dispersed particles immediately invisible. A series of corresponding experiments were accordingly made, in which the " U " tube method was employed, the object being to find out whether actual kataphoresis of the "salt-globulin" did or did not take place in an electric field.

This was the method used by Hardy [1905] whose conclusion that "salt-globulin" was without electric charge has already been referred to. In his experiments, however, as was pointed out by Michaelis [1909] the protein solution may not have been adequately protected from the influence of the acid and alkali produced at the electrodes by electrolysis of the salt present.

In order to avoid all complications due to electrolysis five U tubes were arranged in series in the present instance. The "salt globulin" occupied the centre tube, which was connected by a three-way tap on either side for convenience in filling. The other four tubes, two on either side, contained a solution of the same electrolyte in the same concentration as that used to disperse the euglobulin. All five tubes were fastened by brass·clips to

a stand, so constructed that their position could be altered until the height of liquid in all the tubes was accurately adjusted to a standard level. Only after this was accomplished were the taps opened, and the euglobulin solution placed in contact with the other tubes. Control experiments showed that under these circumstances there was no transference of liquid from one tube to another. The electrodes were placed in the further arms of the two end tubes and the globulin solution was thus securely protected from the influence of any acid or alkali produced there by electrolysis. Litmus and phenol-phthalein were added to the tubes containing the positive and negative electrodes respectively and in all cases the experiment was discontinued long before there was any danger of acid or alkali reaching the centre tube. The resistance of this arrangement was very great, and, although the electrodes (small strips of platinum foil) were connected with the lighting circuit (200 volts) the current which passed amounted to only 0·0001 to 0·005 ampères according to the nature and concentration of the electrolyte employed. At the close of the experiment, which usually lasted from 5 to 10 hours, the contents of the nearer arms of the U tubes adjacent to the globulin tube were tested for the presence of protein by addition of Esbach's reagent or otherwise.

The results are set out in Table IV below, and, in general, confirm the results of Table III. In the case of calcium chloride, barium chloride and

TABLE IV.

Electrical properties of euglobulin, dispersed by various electrolytes.
(U-tube method.)

Protein content = 0·16 %.

Exp. No.	Salt	Concentration, in terms of normality	Electric field, volts.	Current, ampères	Duration of experiment, hours	Sign of electric charge upon protein	Appearance of solution	Sign of electric charge, observed by microscopic method
1	Na₂SO₄	0·05	200	0·0025	8·5	0	Clear solution containing some particles undissolved.	– (agglutinated particles)
2	CaCl₂	0·05	,,	0·002	5·5	0	Almost clear solution, containing some particles.	0
3	BaCl₂	0·04	,,	0·005	5·0	0	Almost clear solution, faint opalescence.	0
4	La(NO₃)₃	0·005	,,	0·0001	5·5	+	Opalescent solution	+
5	Na₃Cit	0·003	,,	0·001	5·0	–	Opalescent solution	
6	,,	0·05	,,	0·0015	9·75	0	Clear solution containing some agglutinated particles.	

sodium sulphate in concentrations of 0·05, 0·04 and 0·05 normal respectively, no migration of the dissolved protein was demonstrated. With the last salt, however, the negative charge carried by the undissolved particles was again shown by the settling which took place less rapidly in the "positive" than in the "negative" arm of the centre U tube, showing that in the latter case the action of gravity was reinforced by motion of the particles towards the positive pole.

Euglobulin dispersed by a small concentration of lanthanum nitrate (= 0·005 normal) displayed a marked positive charge and in the case of sodium citrate[1] a negative charge was shown under similar conditions (concentration = 0·003 normal). If the concentration of sodium citrate was increased to 0·05 normal, however, the dispersed euglobulin showed no migration at all.

The case of sodium citrate is a very interesting one, as the character of the dispersion obtained by employing these two concentrations was also quite different, conforming respectively to the "electrical" and "molecular" types of solution described above (p. 324). With the dilute salt the dispersion is to an opalescent solution and is accompanied by the acquisition of a negative charge; in the stronger solution of citrate, the protein is uncharged and the liquid shows a small precipitate suspended in a clear fluid.

The change from the one type of solution to the other can be seen if a series of solutions be made up containing equal amounts of euglobulin and a concentration of sodium citrate ranging from 0·001 to 0·05 normal. Dispersion is already well marked at 0·001 normal and continues through various grades of diminishing opalescence to a concentration of about 0·003 normal. At a concentration of 0·005 normal the opalescence is greater and at 0·01 normal, a distinct precipitate can be seen. In higher concentrations a gradual clearance takes place, but the solution is now of a different character, less and less of the precipitate remaining undissolved in an otherwise clear solution. In those experiments the protein content was 0·16 %.

Another interesting experiment is the following: The U-tube apparatus was arranged so that in the centre tube was a dispersion of euglobulin in 0·003 N sodium citrate and in the side tubes 0·05 N sodium chloride. As the current passed a precipitation took place in the negative arm of the centre tube, the zone of which continually progressed towards the positive arm. This was followed by a zone of clear solution, also moving in the same

[1] These citrate solutions were carefully prepared to be quite neutral and the hydrogen ion concentration was approximately 10^{-7} normal.

direction. The interface between the advancing chlorine ions and the citrate ions will not be sharply defined, as the former travel more rapidly and tend to over-run and intermix with the latter. A possible explanation is therefore that as both citrate and chlorine ions move towards the positive pole, the latter replace the former in the solution around the protein which is also moving towards the positive electrode, but at a slower rate than either. In low concentration chlorine ions are unable to disperse globulin and precipitation occurs, to be followed again by solution when the concentration of the chlorine ions is sufficiently increased.

The conclusion to be drawn from these experiments is that solution of euglobulin by neutral salts, in case of the more ordinary electrolytes, is due to the formation of some unionised and uncharged compound of salt and globulin—whether by a molecular union (Hardy) or as the result of adsorption (Schryver) there is not, at present, enough experimental evidence to decide. At the same time the phenomenon of dispersion by salts, at any rate in its beginning, is due to an electric charge being deposited on the particles of the euglobulin suspension by the agency of the ions of the electrolyte. The influence of salts upon dispersions of euglobulin by acids and alkalies, dealt with in the next section, is in support of this view.

In the case of the commoner salts, containing only mono- or divalent ions, the electric charges brought into play are not powerful enough to disperse the euglobulin until the concentration is increased to a point where the second ("molecular") type of solution takes place. With such electrolytes the first or "electrical" type of solution as a rule is negligible. It was, however, detected in the case of sodium chloride and sodium sulphate. With sodium citrate, on the other hand, the one type was seen to give place to the other as the concentration of salt was progressively increased.

When euglobulin is denatured by heat, it loses its characteristic property of forming electrically neutral solutions with electrolytes. For example, after heating a dispersion of euglobulin in 0·05 normal sodium sulphate, in which no kataphoresis of the protein could be demonstrated, the protein particles were found to migrate to the positive pole in an electric field. At the same time an alteration took place in the appearance of the solution—the degree of dispersion was diminished and a thick opalescence was developed. With dispersions in sodium chloride (0·1 normal) and calcium chloride (0·15 normal) almost complete agglutination took place on heating.

2. Alteration in electrical conductivity during solution of euglobulin by electrolytes.

It is clear that if a molecular union or an adsorption compound is formed during solution of euglobulin by electrolytes, there must be some diminution in electrical conductivity. Hardy [1905, p. 307] states that a loss of conductivity takes place equal to $1\cdot4$ % to $2\cdot4$ % in case of solution by magnesium sulphate and sodium chloride respectively.

The results of a special set of experiments, made for the purpose, confirm the result of Hardy.

The same sample of euglobulin was used as for the previous set of experiments, and the mixtures when prepared contained $0\cdot6$ % protein. The conductivity was determined:

(1)   of the " salt-globulin " solution,

(2)   of an equal concentration euglobulin suspension in distilled water.

In the case of (1) and (2) the solutions were allowed to settle or were centrifuged and the conductivity measured in the supernatant liquid.

(3)   of an equal concentration salt solution in distilled water.

The conductivity of the distilled water used was found to be negligible in comparison; hence direct comparison was made between (1) and the sum of (2) and (3). All determinations were made at $18°$.

The results are set out in Table V and show a loss of conductivity in every case investigated. The salts used were the chlorides of barium and sodium, sodium citrate and lanthanum nitrate. In the case of what I have termed the " electrical " type of dispersion the diminution in conductivity was proportionally greater than with the molecular type; in the former case it occurred with iso-electric euglobulin to the extent of $13\cdot6$ % and $11\cdot6$ % in case of dispersion by weak (0·004 normal) lanthanum nitrate and sodium citrate respectively, see Exp. 1. With more concentrated salt, 0·05 normal, the loss varied from $1\text{–}4$ % in the case of the four salts employed.

3. Relation of the amount dissolved by a salt to the total amount of euglobulin present.

Notice has already been made of the observation of Mellanby [1905, p. 342] confirmed by Hardy [1905, p. 310] that the amount of euglobulin dissolved by a given concentration of salt is, within certain limits, approximately proportional to the concentration of protein in the original suspension.

In the case of the salt used by Mellanby (sodium chloride in concentration

TABLE V.

Change in electrical conductivity on solution of euglobulin by electrolytes.

Concentration of protein $= 0.6$ %, temperature 18° C.

Exp. No.	Condition of euglo-bulin sus-pension	Salt	Concentration of salt, in terms of normality	Conductivity, in reciprocal ohms				Percentage loss in conductivity	Concentration of euglobulin in solution as "Salt-globulin," %
				Salt solution	Euglobulin suspension	"Salt-globulin" (found)	"Salt-globulin" (calculated)		
1	Approxi-mately iso-electric	NaCl	0·05	0·1389	0·00050	0·1378	0·1394	1·17	—
		BaCl₂	0·05	0·1368	0·00126	0·1368	0·1381	0·99	—
		Na₃Cit	0·004	0·005489	0·00046	0·005331	0·005949	11·6	—
		,,	0·05	0·05289	0·00046	0·05236	0·05335	1·90	—
		La(NO₃)₃	0·004	0·006785	0·000345	0·006277	0·007130	13·6	—
		,,	0·05	0·06708	,,	0·06536	0·006742	3·14	—
2	Alkaline	Na₃Cit	0·004	0·005562	0·000569	0·005578	0·006131	9·93	0·28
		,,	0·05	0·05357	,,	0·05337	0·05414	1·45	0·41
		La(NO₃)₃	0·004	0·006845	,,	0·006482	0·007414	14·4	0·34
		,,	0·05	0·06681	,,	0·06511	0·06738	3·5	0·51
3	Acid	Na₃Cit	0·004	0·005541	0·002145	0·006907	0·007686	11·3	0·07
		,,	0·05	0·05359	,,	0·05415	0·05573	2·93	0·42
		La(NO₃)₃	0·004	0·006850	,,	0·008264	0·008995	8·85	0·45
		,,	0·05	0·06794	,,	0·06717	0·07008	4·33	0·53

from 0·04 to 0·09 normal) solution is of the "molecular" type. On the assumption that this solution is the result of a molecular union between salt and protein (Hardy) it is difficult to explain the existence of the above relationship. On the other hand, if Schryver's view of the salt-solution of euglobulin be accepted in one respect, that is to say if we consider the second or "molecular" type of globulin to be the result of "adsorption" of the salt as a whole, the amount of globulin dispersed by a given concentration of salt might be approximately proportional to the extent of adsorbing surface i.e. to the concentration of euglobulin[1].

It was also possible, however, that solution-rate might be conditioned by the *size* of the euglobulin particles (of which there would be every variety in such a suspension as that used by Mellanby) and that in the time of experiment, final equilibrium had not been maintained but only the particles of small size and comparatively large surface had been successfully attacked.

[1] The experimental support for this view is based upon the fact that the globulin-dissolving capacity and surface-tension (against air) of series of solutions of similar salts, were found to be inversely related to one another, which is the result that should obtain, in accordance with the Willard Gibbs hypothesis. The application of surface tension measurements of air against solution to the case of the protein against solution is not, however, without risk of error.

Some experiments were made to examine this theory, using equal volumes of a 3 % suspension of globulin and 1/10th normal sodium chloride solution. It was found, however, that almost perfect equilibrium was attained within a few minutes after mixing. Experiments were also made, using in the one case finely divided euglobulin and in the other a similar suspension of globulin previously aggregated by freezing; the amount of protein dissolved in the second case by 0·05 normal sodium chloride, under similar conditions, was about 20 % less than in the former. A phenomenon of this magnitude is, in itself, inadequate to explain the relationship observed by Mellanby and Hardy; it may, however, be a contributing factor.

The facts would appear to be best met by some such conception as the following: Solution of euglobulin by such electrolytes as sodium chloride or sodium sulphate, is due to the formation of a " soluble" compound of the globulin and the salt, which is electrically neutral, and to prevent the dissociation of which a large excess of the salt is necessary. Under such circumstances the final equilibrium, i.e. the relative proportion of "dissolved" and "undissolved" globulin, will largely depend upon the concentration of the salt employed and show an approximate constancy if the latter is maintained constant. In other words, the amount of dissolved euglobulin will be roughly proportional to the original concentration (Mellanby).

IV. The effect of Neutral Salts upon Acid and Alkaline dispersions of Euglobulin.

The influence of salts in causing precipitation of euglobulin previously dissolved in acid and alkali is analogous to the corresponding action with denatured serum proteins.

Hardy [1905, p. 317] has drawn attention to the fact that the precipitating action[1] of a salt upon "acid or alkali-globulin" is due to one only of its ions, viz.: that which carries a charge opposite in sign to that carried by the protein, and that the higher the valency of this ion, the greater is the power of the salt.

These results have been confirmed by me and I have also been able to show that, if the concentration of salt is further increased, dispersion will again occur, the particles now taking a charge whose sign is determined by that of the more potent ion of the electrolyte employed. Thus the addition of an appropriate electrolyte to an alkaline or acid solution of euglobulin

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[1] Mellanby [1905] has dealt with the precipitation of globulin from its solution in electrolytes. This is a different phenomenon and a high concentration of salt is required (salting out).

may, after first precipitating the protein, cause the particles to disperse again with a charge opposite in sign to that originally carried.

For example, an alkaline dispersion of euglobulin containing 0·016 % protein, with the particles negatively charged, was precipitated by the addition of lanthanum nitrate to a concentration of 0·001 normal; at a concentration of 0·008 to 0·01 normal, dispersion again took place, the particles now being positively charged. In a similar experiment with sodium chloride the precipitation took place at a concentration of salt equal to 0·02 normal; at 0·05 normal the globulin was again dispersed, this time bearing a negative charge.

These results present a close analogy with what takes place in case of denaturated serum-proteins, where the degree of dispersion in acid and alkaline solution is also greatly influenced by the presence of neutral salts, and in two ways. In the first place the reaction of protein-containing solutions, whether denaturated or not, becomes altered on addition of neutral salts [Chick and Martin, 1911, p. 21; 1912, p. 280], being shifted in the direction of the neutral point. In acid solution, the concentration of hydrogen ions is lowered and the concentration of hydroxyl ions lessened if the solution be alkaline; the effect is related to the valency of the anion and kation respectively of the electrolytes in the two cases. In the second place, the electric charge carried by the protein particles is modified and may be lessened or even changed in sign by the addition of electrolytes if of opposite sense to that carried by the more potent ion of the electrolyte added.

The effect of the above-mentioned salts in modifying the reaction[1] of solutions containing protein has also been demonstrated in the case of euglobulin (see Tables I, VI and VII). It was therefore necessary to determine in how far the precipitating effect of salts was due to this effect. It is evident that solutions either too acid or too alkaline for precipitation of euglobulin might be adjusted to the iso-electric reaction by the addition of an appropriate electrolyte, and indeed this frequently occurred. For example (Experiment 12, Table VI), addition of sodium citrate to a concentration of 0·002 N, caused precipitation of an acid dispersion of euglobulin (0·016 % protein) at the same time reducing the concentration of hydrogen ions to a point very near the iso-electric point for this protein. In some cases the change of reaction extended to the other side of the iso-electric point, e.g. Table VI, Experiment 13; in this case the observed change of sign in electric charge taking place simultaneously with dispersion by sodium citrate, could be explained on the ground of change of reaction alone. By comparison of

[1] The phenomenon in absence of protein is perceptible but negligible.

Experiment 7 with 12 and 13, Table VI, this change in reaction is seen to be increased proportionally with the degree of valency possessed by the ions of the salt employed.

TABLE VI.

The effect of sodium sulphate and citrate upon an acid dispersion of euglobulin (0·016 %).

Exp. No.	Salt added	Concentration of salt in terms of normality	Cc. N/100 HCl (or equivalent) added in total volume of 10 cc.	Concentration of hydrogen ions, in terms of normality	Sign of electric charge carried by the particles	Degree of agglutination
1	—	—	0·0		−	Partial agglutination.
2		—	0·075		−	Agglutinated completely.
3		—	0·1		−	,,　　　　　,,
4		—	0·2	$10^{-4.10}$ (810×10^{-7})	+	Dispersed, clear solution.
5		—	0·3		+	,,　　　,,　　　,,
6		—	0·7		+	,,　　　,,　　　,,
7	Na_2SO_4	0·03	0·5	$10^{-4.45}$ (357×10^{-7})	−	Agglutinated.
8		0·04	,,		−	Partial agglutination.
9		0·05	,,		−	Dispersed.
10		0·07	,,		0	,,
11	Na_3Cit	0·001	0·7		+	Dispersed.
12		0·002	,,	$10^{-5.41}$ (39×10^{-7})	−	Almost complete aggluti-
13		0·003	,,	$10^{-6.04}$ ($9·2 \times 10^{-7}$)	· ·	Dispersed.　　[nation.
14		0·004	,,		−	Dispersed, faintly opalescent solution.

In many cases, however, acid and alkaline solutions of euglobulin, with their positively and negatively charged particles respectively, were first precipitated and afterwards dispersed, the particles bearing an electric charge of changed sign in solutions whose reaction still remained more acid and more alkaline respectively, than the iso-electric point[1]. A good example of this is seen in Table I, Experiment 9, where addition of 0·05 N sodium sulphate to an acid dispersion of euglobulin (0·032 % protein) caused the particles to be dispersed and to carry a negative charge in a solution where the hydrogen ion concentration, equal to 1590×10^{-7} normal, was far on the acid side of the iso-electric point. Experiment 7 of Table II is another instance of the same phenomenon[1].

With an alkaline suspension of euglobulin a corresponding series of results was obtained with lanthanum nitrate. The results are set out in

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[1] In higher concentration of sodium sulphate, the dispersed globulin appears to be electrically neutral, see Exp. 10, Table VI.

Table VII. A preliminary set of experiments (1 to 8), in absence of electro-lytes, showed a positive charge to be acquired by the protein particles at a concentration of hydrogen ions equal to 204×10^{-7} normal. In the alka-line suspension used, the concentration of hydrogen ions was equal to 0.0013×10^{-7} normal. In presence of 0.006 normal lanthanum nitrate the

TABLE VII.

Effect of lanthanum nitrate upon an alkaline dispersion of euglobulin.

Protein $= 0.016$ %.

Exp. No.	Salt added	Concentration of salt, in terms of normality	Cc. N/100 HCl (or equivalent) added in a total volume of 10 cc.	Cc. N/100 NaOH (or equivalent) added in a total volume of 10 cc.	Concentration of hydrogen-ions, in terms of normality	Sign of electric charge carried by the particles	Degree of agglutination
1	—	—	0·2	—			Dispersed, clear solution.
2	—	—	0·1	—	$10^{-4.69}$ (204×10^{-7})	+	Dispersed, faintly opalescent solution.
3	—	—	0·05	—	$10^{-7.00}$ (1.05×10^{-7})	−	Agglutinated.
4	—	—	0	—		−	Dispersed, opalescent sol.
5	—	—	—	0·5			,, ,, ,,
6	—	—	—	0·1			Dispersed, clear solution.
7	—	—	—	0·2			,, ,, ,,
8	—	—	—	0·5	$10^{-9.88}$ (0.0013×10^{-7})		,, ,, ,,
9	La(NO₃)₃	0·0005	—	0·5			Dispersed, opalescent sol.
10		0·001	—	,,			Agglutinated.
11		0·002	—	,,		+	Agglutinated almost completely.
12		0·004	—	,,		+	,, ,, ,,
13		0·005	—	,,		+	Agglutination not complete.
14		0·006	—	,,	$10^{-7.63}$ (0.24×10^{-7})	+	,, ,, ,,
15		0·008	—	,,			Dispersed partly.
16		0·01	—	,,			,, ,, ,,
17		0·02	—	,,			,, ,, ,,

alkalinity was reduced almost to the neutral point. At the same time the charge on the particles was found to be positive in a solution whose reaction was on the alkaline side of the iso-electric point, and where, in the absence of any electrolyte, the particles would be negatively charged.

In these instances the effect of electrolytes recalls the exactly similar set of phenomena obtaining in the case of denaturated serum-proteins mentioned

above, and also the analogous influence of salts which has been observed
in case of inorganic colloidal solutions [Burton, 1909]. In certain of the
latter there has been demonstrated a selective adsorption of the ion bearing
a charge opposite in sign to that of the charged colloidal particles [Linder
and Picton, 1895; Whitney and Ober, 1902; Freundlich, 1910] and this
explanation may be extended to the case of proteins.

In this connection, certain of the conductivity determinations in Table V,
i.e., those of Experiments 2 and 3, are of special interest. These experiments
were made with suspensions of euglobulin which were slightly on the acid
and alkaline side, respectively, of the iso-electric point. The effect of adding
electrolytes containing trivalent positive $(La(NO_3)_3)$ and negative (Na_3Cit)
ions was carefully studied. The loss of conductivity was found to be greater
for sodium citrate if the globulin suspension were originally acid (i.e. charge
on the protein particles positive) and greater for lanthanum nitrate if the
globulin suspension were alkaline. At the same time, the degree of dis-
persion was measured by the content of protein in the supernatant fluid
after centrifuging (see last column), and was found to be less. In all cases
equivalent solutions of the salts were compared and the effects were more
marked in the experiments with less concentrated salt (0·004 normal), in
which case dispersion has been shown to be of the "electrical" type.

Analogy with euglobulin presented by caseinogen and other proteins.

Caseinogen has been found to show close analogy with euglobulin as
regards the effects of electrolytes upon its solutions in either acid or alkali.

Michaelis and Rona [1910, 2] drew attention to the fact that among the
naturally occurring proteins whose solution was accompanied by the acquisi-
tion of electric properties, in addition to globulin, were caseinogen, gliadin,
and edestin, and they determined the iso-electric point in each case. The
result was especially interesting in the case of caseinogen, where the
particles were found to be iso-electric with the solution, and to be precipitated
when the concentration of hydrogen ions was $1·8 \times 10^{-5}$ normal, a degree
of acidity far beyond that determined for euglobulin or for the heat-
denatured proteins of serum.

The following experiments, set out in Table VIII, show the analogy with
euglobulin to be closely maintained in respect also of the action of electrolytes.
For example, in an opalescent solution (0·05 %) in weak hydrochloric acid,
in which the protein particles were yet visible under the microscope using
a high power and dark ground illumination, the caseinogen was found

to be positively electrified. This solution was readily precipitated by a minute concentration of sodium sulphate (equal to 0·0005 normal) or sodium citrate (0·00005 normal); with increased concentration of either salt (0·015 and 0·001 normal, respectively) the protein particles were again dispersed and found to carry a negative charge in both cases, see Table VIII, Experiment A.

TABLE VIII.

Influence of electrolytes upon agglutination of caseinogen (Merck's pure casein).

(A) Dispersed with a little HCl.   (B) Dispersed with a little NaOH.

× × ×   Complete agglutination.   ×   Partial agglutination.
    × ×   Almost complete agglutination.   × −   Almost complete dispersion.
                                −   Complete dispersion.

Exp. No.	Concentration of protein, %	Salt added	Concentration of salt, in terms of normality	Degree of agglutination	Sign of electric charge carried by the particles
(A)	0·05	0	0·00	−	+
	,,	Na₂SO₄	0·0005	× × ×	
			0·001	× ×	
			0·005	× ×	
			0·01	× −	
			0·015	−	−
			0·02	−	
	,,	Na₃Cit	0·00001	× ×	
			0·00005	× × ×	
			0·0001	× ×	
			0·0005	×	
			0·001	−	−
			0·002	−	
(B)	0·03	—	0·00	−	−
	,,	CaCl₂	0·01	−	
			0·02	× −	−
			0·05	×	−
			0·09	×	0
			0·10	×	
			0·15	×	
			0·2	× −	0
			0·5	−	
	,,	La(NO₃)₃	0·00002	−	
			0·00005	×	−
			0·00008	×	
			0·0001	× ×	
			0·0002	× × ×	
			0·0005	× −	
			0·001	−	+

Experiment (B) with an alkaline dispersion of caseinogen 0·03 %, showed an exactly analogous set of phenomena with lanthanum nitrate.

With calcium salts the action appears to be different in character. In the first place no complete precipitation takes place; the size of the particles is increased, but no complete agglutination occurs although the solution becomes turbid. The particles, when dispersed again by increased concentration of the salt (0·2 to 0·5 normal) do not appear to carry any electric charge. In this respect also the analogy with both denatured serumproteins and with euglobulin is maintained.

V. Summary.

1. The iso-electric point for euglobulin has been re-determined and found to coincide with the point of most rapid agglutination, viz.: at a hydrogen ion concentration of about 3×10^{-6} normal, a figure which agrees with that obtained by Michaelis and Rona [1910, 2].

2. The solution or dispersion of euglobulin by electrolytes is shown to be much influenced by the nature (especially as regards valency) of the constituent ions and to be of two general types:

(a) "electrical" type of solution in which the euglobulin dispersion is accompanied by the acquisition of an electric charge by the protein particles, the sign of which is similar to that of the more potent ion of the electrolyte employed.

(b) "molecular" type of solution, in which the dissolved euglobulin is electrically neutral.

In type (a) the dispersion is considered to result from a specific adsorption of the ion possessing the higher valency, in (b) from a molecular union with (Hardy) or adsorption of (Schryver) the salt as a whole. Both types of solution are accompanied by loss of electrical conductivity in the liquid.

The "electrical" type of solution is well seen in case of dispersion by such salts as sodium citrate and lanthanum nitrate in low concentration; in case of the more ordinary salts, containing mono- or divalent ions only, the electric forces concerned are not powerful enough to disperse globulin until the concentration is raised to a point where "molecular" solution takes place. In the case of sodium citrate, the "electrical" type of solution was found to change to the "molecular" type as the concentration of the salt was increased.

3. Euglobulin, when denatured by heat, no longer possesses the property of forming the "molecular" type of solution with electrolytes. On

heating the latter, in some cases the degree of dispersion is merely diminished, and the protein particles acquire an electric charge, whose sign is determined by the more potent ion of the electrolyte employed; in other cases agglutination takes place.

4. The reaction of acid and alkaline solutions of euglobulin is greatly influenced by the addition of electrolytes, the hydrogen and hydroxyl ion concentration being reduced respectively. In case of the former the effect is much increased with rising valency of the anion and in alkaline solution the result is determined by the valency of the kation.

5. The influence of electrolytes in causing precipitation of globulin dissolved in acid and alkali may, in some instances, be adequately explained by the alteration in reaction, described under 4; in this way solutions too acid or too alkaline for agglutination of the globulin may be adjusted to the iso-electric point by the addition of an appropriate electrolyte.

Precipitation by electrolytes may, however, also take place in solutions whose reaction is still far removed from that of the iso-electric point. In these instances it is attributed to neutralisation of the electric charge originally carried by the protein particles by means of a specific adsorption of the oppositely charged ion of the electrolyte; the effect is related to valency.

6. In the properties regarding solution and precipitation detailed under 1, 4 and 5, euglobulin, in common with caseinogen, and the vegetable globulins presents a very interesting analogy with heat-denaturated proteins. Euglobulin differs from heat-denaturated protein in its capacity to form solutions with electrolytes in which the protein particles are electrically neutral.

In conclusion, I wish to express my indebtedness to Prof. C. J. Martin for much helpful advice and criticism.

REFERENCES.

Abderhalden (1903), *Zeitsch. physiol. Chem.* **37**, 49
—— (1904), *Zeitsch. physiol. Chem.* **44**, 17.
Burton (1909), *Phil. Mag.* (6) **17**, 583.
Chick and Martin (1911), *J. Physiol.* **43**, 1.
—— —— (1912), *J. Physiol.* **45**, 261.
Dabrowski (1912), *Bull. Akad. Sci. Cracow* A, June, 485.
Freundlich (1910), *Zeitsch. physikal. Chem.* **73**, 385.
Hardy (1900), *Proc. Roy. Soc.* **66**, 110.
—— (1905), *J. Physiol.* **33**, 251.
Linder and Picton (1895), *J. Chem. Soc.* **67**, 63.

Mellanby (1905), *J. Physiol.* 43, 338.

Michaelis (1909), *Biochem. Zeitsch.* 19, 181.

—— and Mostynski (1910), *Biochem. Zeitsch.* 24, 79.

—— and Rona (1910, 1), *Biochem. Zeitsch.* 27, 38.

—— —— (1910, 2), *Biochem. Zeitsch.* 28, 193.

Moll (1904), *Beiträge*, 4, 563.

Osborne and Harris (1905), *Amer. J. Physiol.* 14, 151.

Pauli (1899), *Pflüger's Archiv.* 78, 315.

Pfeiffer and Modelski (1912), *Zeitsch. physiol. Chem.* 81, 329.

Schryver (1910), *Proc. Roy. Soc.* B, 83, 96.

Sörensen and Jürgensen (1911), *Biochem. Zeitsch.* 31, 397.

Starke (1900), *Zeitsch. Biol.* 40, 494.

Whitney and Ober (1902), *Zeitsch. physikal. Chem.* 39, 630.

XXXIII. THE FAT OF YEAST.

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Probably owing to the small quantity of material usually available, only one previous examination of yeast fat, that by Hinsberg and Roos [1903], appears to have been carried out, and, as this investigation was inconclusive on several points, it appeared to be worth while to go over the ground again. In an examination of yeast products for the "vitamines" or "hormones" connected with the phenomenon of animal growth, fairly large quantities of the crude fat of yeast became available and on this the following investigation was carried out. The results confirm the statement of the above mentioned workers that the principal saturated fatty acid present is a pentadecoic acid of empirical formula $C_{15}H_{30}O_2$, but in addition the author separated a small quantity of arachidic acid, $C_{20}H_{40}O_2$. There might be noted here the diversity of opinion in the literature as to the existence of a pentadecoic acid in the natural fats and the widely different values given to the physical constants of this acid by those authors who claim to have isolated it. The values given for the melting point vary from 70° to 52°, while the same constant for the methyl ester varies from 68° to 38°. Probably some of the acids described were impure as it is unlikely that a sufficient number of branched chain acids occur in the natural fats to account for the varying values given. The highest melting point obtained by the author for this acid was 59° while Hinsberg and Roos give 57°. An important point to be noticed is that for the synthetic normal acid of this composition, synthesised by Krafft [1879] and also by Le Sueur [1905], the melting point given is in both cases lower than the one found for the yeast acid, being stated as 51° by the former and 53° by the latter worker. The melting points of the simpler derivatives also disagree with those of the corresponding derivatives of the synthetic acid. The work was started in the belief that the substance was probably a mixture, but all attempts to separate it into more than one substance were unavailing and the only

conclusion possible was that the substance was really a single acid of the composition $C_{15}H_{30}O_2$ and, at the same time, that it was not the normal pentadecoic acid.

In dealing with the unsaturated acids the author experienced considerable difficulty in directly isolating any pure product, the simpler derivatives being difficult to deal with and indicating no satisfactory means of separation. By oxidation with potassium permanganate, however, very satisfactory specimens of the corresponding di- and tetra-hydroxy acids were obtained and these showed the presence in the original fat of the acids $C_{16}H_{30}O_2$, $C_{18}H_{34}O_2$ and $C_{18}H_{32}O_2$. Hinsberg and Roos obtained results which they interpreted as showing the presence of the acids $C_{12}H_{22}O_2$ and $C_{18}H_{34}O_2$ though they themselves query these formulae.

The phytosterol obtained melted at 145–147° and was apparently identical with that obtained by the other workers from one sample of beer yeast. The compound melting at 159° obtained by them from the majority of yeast samples appeared not to be present in the sample under examination.

Preliminary Examination.

The crude fat was hydrolysed with alcoholic potash and after the alcohol had been distilled off the crude dry soap was extracted with ether. From the ethereal solution the yeast cholesterol was obtained. The crude soap was then redissolved in water and after neutralisation of excess of alkali, the fatty acids were precipitated in the form of their lead salts and the usual rough separation of the salts of the saturated and unsaturated acids made by extraction with ether. The free acids were then liberated from their lead salts by hydrochloric acid and treated as described below.

The Saturated Fatty Acids.

When the dark brown solid mass, obtained from the ether-insoluble lead salts, was distilled under a pressure of 100 mm. by far the larger part passed over between 250° and 260°. This was redistilled and came over at about 215° under 15 mm. pressure. In the first distillation the process was not pressed to an extreme extent and the residue left in the flask was purified by crystallisation only. The portion which had been twice distilled was crystallised several times from methyl alcohol and then melted at 54–55°. An iodine absorption determination gave however an iodine value of 29·5, and, as this was only very slowly altered by crystallisation, the acid was dissolved in dilute potash solution and treated with 1·5 per cent. permanganate

solution so as to convert the impurities consisting of unsaturated acids into the corresponding hydroxy-acids. The solution having been treated with sulphur dioxide, the precipitated acids were filtered off and treated with a small quantity of ether. Filtration from the hydroxy-acids and crystallisation of the substance obtained from the ethereal solution gave a white crystalline body, melting at 59° and showing no iodine absorption when treated with Hübl's solution.

Pentadecoic Acid.

On analysis the substance, whose preparation has just been described, gave the following result:

$$0.1686 \text{ g.}; \quad 0.4611 \text{ } CO_2; \quad 0.1878 \text{ } H_2O.$$
$$\text{Found } 74.59 \text{ } \% \text{ C}; \quad 12.38 \text{ } \% \text{ H.}$$
$$\text{Calc. for } C_{15}H_{30}O_2 \text{ } 74.38 \text{ } \% \text{ C}; \quad 12.39 \text{ } \% \text{ H.}$$

This substance was evidently the same as that separated by Hinsberg and Roos and described by them as being definitely pentadecoic acid.

A determination of the molecular weight by the freezing point method showed that 0.4310 g. substance dissolved in 24.2174 g. glacial acetic acid gave a depression of the freezing point of 0.295°; whence molecular weight = 235. Calculated for $C_{15}H_{30}O_2 = 242$. On titration with alcoholic potash 1.8344 g. acid took 14.9 c.c. N/2 KOH for neutralisation.

$$\text{KOH required} = 22.74 \text{ } \% \text{ weight of acid.}$$
$$\text{Calc. for } C_{15}H_{30}O_2 = 23.14 \text{ } \% \text{ weight of acid.}$$

The silver salt, prepared by precipitating a neutral solution of the potassium salt with silver nitrate, was obtained as a white amorphous precipitate and gave the following result on ignition:

$$0.4288 \text{ g. salt}; \quad 0.1308 \text{ g. metallic silver.} \quad \text{Molecular weight of the acid} = 247.$$
$$\text{Calc. for } C_{15}H_{30}O_2 = 242.$$

The above estimations point to the substance being pentadecoic acid or a mixture with practically the same composition. If not a single compound the substance was most likely to be a mixture of two of the three acids, myristic, palmitic and stearic acid. Referring to the determinations by Heintz of the melting points of mixtures of any two of these acids it was found that with a melting point of 59 the substance might be a mixture of (a) 15 % myristic with 85 % palmitic acid, (b) 40 % myristic with 60 % stearic acid, (c) 45 % palmitic with 55 % stearic acid. Assuming the substance to be one of these mixtures, weighed quantities of pure myristic, palmitic or stearic acids were added and the melting point curves plotted. In no case did the curve agree with that for any pair of the above three acids. The melting point for a

mixture of all three of the above acids lies below that actually determined. Other methods for the separation of a possible mixture in the substance were tried but without result. Thus the fractional precipitation of the magnesium salts and the continual crystallisation of the lithium salts from alcohol were without effect. To obtain further evidence of the homogeneous nature of the substance several derivatives were prepared and compared with the corresponding compounds of the better known fatty acids.

The *methyl ester*, prepared by passing gaseous hydrochloric acid into a mixture of the acid and methyl alcohol, was obtained as a white crystalline substance which could be crystallised from a small quantity of methyl alcohol and melted at 26°, which is considerably lower than the melting point for either methyl palmitate or methyl stearate.

The *anilide* was prepared by boiling the acid for some hours with excess of aniline. It crystallised from alcohol in pearly scales and melted sharply at 86–87°.

Myristic anilide melts at 84°, palmitic anilide at 90·5° and stearic anilide at 94°.

Analysis :   0·2117 g.; 10·3 cc. moist N at 16° and 772 mm. N=4·48 %.
                     Calc. for $C_{21}H_{35}ON$ N=4·41 %.

The *amide* was prepared by heating together equal quantities of phosphorus pentachloride and the acid in chloroform. The solvent and phosphorus oxychloride were distilled off under slightly reduced pressure and the residue poured into strong ammonia. It crystallised in small flakes from 50 % alcohol and melted at 94–95°.

The amide of the synthetic pentadecoic acid, prepared by Le Sueur, melted at 102·5°, while palmitic amide melts at 107°.

0·1443 g.; 7·0 cc. moist N at 15° and 760 mm.   N=5·71 %.
Calc. for $C_{15}H_{31}ON$ N=5·81 %.

The whole of the evidence points to the substance being a definite compound of the composition $C_{15}H_{30}O_2$, but at the same time being not identical with the normal synthetic acid of this composition.

Arachidic Acid.

It has been mentioned that a small quantity of material remained behind in the flask when the pentadecoic acid was distilled under reduced pressure. This small quantity of material was several times crystallised from alcohol and was finally obtained as a white crystalline body which melted sharply at 77°.

On analysis it gave the following results :

<div align="center">

0·1212 g. ; 0·3440 g. CO_2 ; 0·1385 g. H_2O.

$C = 77·40 \, \%$, $H = 12·70 \, \%$.

Calc. for $C_{20}H_{40}O_2$ $C = 76·92 \, \%$, $H = 12·82 \, \%$.

0·4585 g. required 14·2 cc. N/10 KOH for neutralisation.

KOH required $= 17·34 \, \%$ of weight of acid.

Calc. for $C_{20}H_{40}O_2$ 17·95 $\%$ of weight of acid.

</div>

The substance was optically inactive, did not absorb iodine from Hübl's solution and did not react with acetic anhydride. The substance was almost certainly therefore arachidic acid.

<div align="center">

The Unsaturated Acids.

</div>

The unsaturated fatty acids, liberated from the ether-soluble lead salts, formed a dark brown oily mass which behaved as a "semi-drying" oil when exposed in films to the air. Attempts were at once made to purify this mass by distillation under reduced pressure but without much success, as decomposition of some portion took place and the distillation was rendered useless. Distillation in steam was also of little avail the quantity passing over being very small. In an attempt to obtain some separation by means of salts, it was noticed that the barium salts, a sticky mass containing a small quantity of water, were soluble to some extent in benzene, toluene and ether, but almost insoluble in cold alcohol while with hot alcohol the mass yielded an apparently homogeneous glue with the solvent. These observations together with an iodine value of 138 for that portion of the acids whose barium salt was soluble in benzene, point to the presence in the mass of some acids of the linoleic series and the presence of some proportion of these acids would account for the "semi-drying" properties of the oil.

Attempts at direct separation of the mixture having failed, the whole "unsaturated" fraction was neutralised with caustic potash, dissolved in a large volume of water and an equal volume of 1·5 per cent. potassium permanganate solution run into it. After standing for ten minutes sulphurous acid solution was added and the precipitated acids filtered off. The filtrate was found not to contain any appreciable quantity of hydroxy-acids, thus showing the absence in the original mixture of any appreciable quantity of acids of the linoleic series.

The acids precipitated by sulphur dioxide were washed with small quantities of ether to remove unoxidised material and afterwards treated with large quantities of the same solvent. The ether-soluble portion was crystallised from alcohol and the purified dihydroxy-acid is described below. The portion insoluble in ether was crystallised from large quantities of water and was identified in the manner hereafter described as a tetrahydroxy-acid.

The Dihydroxy-acid.

This substance was obtained as a well crystallised white powder, insoluble in water, slightly soluble in ether but soluble in hot alcohol. It melts at 124–125°.

Analysis : 0·1568 g. ; 0·3894 g. CO_2 ; 0·1566 g. H_2O.
$$C = 67·73 \text{ } \%, \text{ } H = 11·09 \text{ } \%.$$
Calc. for $C_{17}H_{32}O_2(OH)_2$ $C = 67·55 \text{ } \%, \text{ } H = 11·25 \text{ } \%.$
0·6453 g. required 21·0 cc. N/10 KOH for neutralisation.
$KOH = 18·22 \text{ } \%$ of weight of acid.
Calc. for $C_{17}H_{32}O_2(OH)_2$, $KOH = 18·54 \text{ } \%$ of weight of acid.

The acetyl derivative gave the following result :

0·8212 g. gave acetic acid equivalent to 53·7 cc. N/10 acetic acid.
Acetic acid = 40·45 $\%$ weight of substance taken.
Calc. for $C_{17}H_{34}O_4$ 39·73 $\%$.

The above figures point to the substance being a dihydroxy-acid of the composition $C_{17}H_{34}O_4$ and the melting point and general properties agree exactly with a substance of this composition described by Fahrion [1893]. This substance was shown by Ljubarsky [1898] to be probably a mixture of dihydroxypalmitic acid and dihydroxystearic acid, which mixture the latter author found to behave in many respects as a chemical individual. In view of the fact that an unsaturated acid of the oleic series with the composition $C_{17}H_{32}O_2$ has hitherto not been separated with certainty from natural fats, it seems more probable that the substance separated was the mixture which Ljubarsky claims it to be, than a single chemical individual, although no separation was possible by repeated crystallisation from alcohol.

If this is so the original fat must have contained two unsaturated fatty acids of the oleic series with compositions $C_{16}H_{30}O_2$ and $C_{18}H_{34}O_2$ respectively.

Tetrahydroxy-acid.

The oxidation product insoluble in ether but crystallising from large quantities of water was analysed with the following results :

0·1465 g. ; 0·3330 g. CO_2 ; 0·1380 g. H_2O.
$$C = 61·99 \text{ } \%, \text{ } H = 10·46 \text{ } \%.$$
Calc. for $C_{18}H_{32}O_2(OH)_4$ $C = 62·07 \text{ } \%, \text{ } H = 10·34 \text{ } \%.$
1·0878 g. requires 32·3 cc. N/10 KOH for neutralisation.
$KOH = 16·63 \text{ } \%$ of acid.
Calc. for $C_{18}H_{32}O_2(OH)_4$ $KOH = 16·18 \text{ } \%.$
0·4321 g. when converted into acetyl compound and hydrolysed gave acetic acid equivalent to 50·5 cc. N/10 acetic acid.
Acetic acid obtained = 70·12 $\%$ of weight of acid taken.
Calc. for $C_{18}H_{32}O_2(OH)_4$ 68·96 $\%$.

The substance is a white crystalline body of silky lustre, melting at 156°, soluble in hot alcohol and glacial acetic acid but insoluble in nearly all other solvents. The general properties of the acid, together with the analyses given above, point to the substance being a tetrahydroxy-acid of the composition $C_{17}H_{31}(OH)_4.COOH$. The best known acid of this composition is tetrahydroxystearic acid (sativic acid), formed by the oxidation of linoleic acid, but this melts at 173–174°. Apparently, however, there are several isomeric acids of this composition, for other observers have reported acids melting at 152° and 165° and the acid separated may be identical with one of these. At any rate, the isolation of this substance points definitely to the existence in yeast fat of an unsaturated acid of the linoleic series with the empirical composition $C_{18}H_{32}O_2$, that is, an acid certainly isomeric, but perhaps not identical with ordinary linoleic acid.

Yeast Cholesterol.

The ethereal extract of the crude potassium salts obtained from the first hydrolysis of the yeast fat was worked up in the usual way for the yeast cholesterol. The product obtained, after being crystallised several times from alcohol, melted at 145–147, and this melting point was not altered by repeated crystallisation. It had $[\alpha]_D = -75\cdot54°$ and gave similar colour reactions to the majority of the phytosterols. It was apparently identical with the cholesterol obtained from one sample of yeast by Hinsberg and Roos. The cholesterol, melting at 159°, which these authors most frequently obtained from yeast was not isolated in spite of a careful search being made for it.

Summary.

A comparison of the results obtained by Hinsberg and Roos and by the author is shown in columns I and II respectively of the following table.

I	II
Saturated acid $C_{15}H_{30}O_2$. M.P. 56°.	Saturated acid $C_{15}H_{30}O_2$. M.P. 59°.
Not identified in the mixture.	Saturated acid $C_{20}H_{40}O_2$. M.P. 77°. Arachidic Acid.
Unsaturated acid $C_{12}H_{22}O_2$.	Not identified in the mixture.
Unsaturated acid $C_{18}H_{34}O_2$.	Unsaturated acids $C_{16}H_{30}O_4$ and $C_{18}H_{34}O_2$. Presence shown by their oxidation products.
Not identified in the mixture.	Unsaturated acid $C_{18}H_{32}O_2$, shown by its oxidation product.
Yeast cholesterols melting at 159 and 145–148°.	Yeast cholesterol melting at 145–147°.

REFERENCES.

Fahrion (1893), *J. Soc. Chem. Ind.* 936.
Hinsberg and Roos (1903), *Zeit. physiol. Chem.* **38**, 1.
Krafft (1879), *Ber.* **12**, 1671.
Le Sueur (1905), *J. Chem. Soc.* **87**, 1898.
Ljubarsky (1898), *J. pr. Chem.* [2] **57**, 19.

XXXIV. THE INFLUENCE OF THE CARBONATES OF THE RARE EARTHS (CERIUM, LANTHANUM, YTTRIUM) ON GROWTH AND CELL-DIVISION IN HYACINTHS.

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The effects of various organic and inorganic bodies on growth and cell-division in both plants and animals have been studied by several researchers in this laboratory during the past few years. Moore, Roaf, and Whitley [1905] investigated the action of acids and alkalis on the development of the fertilized eggs of the sea-urchin, *Echinus esculentus.* Their inquiry was directed to the effects of variation in hydrogen and hydroxyl ion concentration in the medium (sea-water) in which the eggs were allowed to develop, and they found that within certain narrow limits of concentration, alkalis favoured cell-division, whereas acids were invariably fatal. Small additions of acids inhibited cell-division and growth, and at a concentration of 0.001 molar practically all cell-division was stopped. A slight increase in alkalinity favoured development, at the same time producing irregularities in cell-division. Beyond the optimum concentration of alkali, exceedingly irregular division, resulting in particular in the production of multi-nucleated cells, was observed. At a concentration of 0.0015 M. of caustic alkali, however, cell-division was completely arrested.

Whitley [1905] observed similar effects on the eggs of plaice, and also noted the action of the indicators phenolphthalëin and dimethylaminoazobenzene on the eggs both of plaice and echinus. He found that while phenolphthalëin was deadly to the eggs of echinus, it was harmless to those of the plaice. On the other hand, the azobenzene derivative quickly killed the latter, and appeared, if anything, to have a favourable effect on the development of the former.

Moore, Knowles, and Roaf [1908] extended the observations to plants, using for their experiments the common hyacinth. They obtained similar

results in regard to the influence of acids and alkalis, and they also showed that the cation had a specific effect, potassium being much more stimulating than sodium to both rootlets and foliage leaves. The phosphatic ion also had a special effect on the flower, causing an increase in size at optimum concentration, and at higher concentrations an irregular inflorescence, with packed florets on a dwarfed stalk.

Histologically they observed depression of nuclear division with acids, and thickening of the cell walls; with alkalis, increased nuclear division, changes in chromosomes and irregular figures, while the cell outlines became obscured.

Coppin [1912] studied the effect of allantoin and other purine derivatives on the growth of hyacinths, and also salts of organic acids such as sodium huminate, sodium malate, and sodium oxalate.

These latter substances, as well as sodium urate, had a stimulating effect on the growth of the hyacinths, but allantoin and the other purine substances inhibited both growth and cell-division.

Working on somewhat different lines, Ransom [1912] observed the action of caffeine upon the germination of seeds. He used a large number of different seeds, and his method consisted in soaking the seeds for a short time in his caffeine solutions before sowing them in the usual manner. He found that caffeine, even in a very dilute solution, had a powerful effect in retarding germination and growth; while a concentration of 1 per cent. in many cases completely inhibited germination.

In the present inquiry, salts of the rare earths were used to test whether these produced any physiological effects on growth and cell-division. For this purpose the carbonates of cerium, lanthanum, and yttrium were selected.

Preparation of carbonates.

The carbonates were prepared, according to Moissan, in the following ways:

(1) Cerium. A solution of cerium nitrate was treated with ammonium carbonate, and the precipitate of cerium carbonate filtered off, and thoroughly washed.

(2) Lanthanum and Yttrium. The hydroxides of the metals were taken, suspended in water, and thoroughly saturated with carbon dioxide over a period of several hours. The precipitates were then filtered off.

Preparation of the carbonate solutions.

Owing to the very slight solubilities of the carbonates of cerium, lanthanum and yttrium, some difficulty was experienced in preparing solutions. Finally two or three grams of each of the respective carbonates were suspended in about two litres of Liverpool tap-water[1], and carbon dioxide was passed into the bottles for one or two hours, thus ensuring complete saturation. After being allowed to stand overnight, the undissolved residues were filtered off.

These clear filtrates were the actual solutions used in the experiments. Their concentrations were estimated by taking a measured volume of each, evaporating to dryness, and weighing the residue. The following figures were obtained:

Cerium	...	...	...	0·007 %
Lanthanum	...	...	...	0·01 %
Yttrium	...	...	...	0·017 %

In all probability they contained a mixture of the respective carbonate and bicarbonates.

Effect on the growth of hyacinths.

The plants used were a common variety of hyacinth. Healthy bulbs of as nearly uniform size as possible were selected. They were placed in hyacinth glasses which had been blackened on the outside with black lacquer to prevent action of light upon the rootlets. The hyacinth glasses held about 450 cc. and the solutions mentioned were filled in until they just wetted the bulbs. In addition, controls were grown under precisely similar conditions in Liverpool tap-water. Water was added to the glasses from time to time to make up for the loss due to evaporation. A few of the ends of the growing rootlets were cut off on the twenty-fifth day for the purpose of studying the effects of the solutions on cell-division and nuclear changes. These were all immediately fixed in Flemming's solution, cut in paraffin, and stained for nuclear figures by Heidenhain's iron-alum, haematoxylin method.

On the twenty-fourth day, a measured volume of the fluid was removed from each of the different solutions, filtered, and evaporated to dryness, organic matter being removed by ashing with ammonium nitrate. The residues were then weighed. Results:

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[1] Liverpool tap-water is very pure surface water and practically free from inorganic salts.

	24th day	Original
Cerium	0·0068 %	0·007 %
Lanthanum	0·0076 %	0·01 %
Yttrium	0·0066 %	0·017 %

Thus in the case of the cerium carbonates there had been little or no absorption; but in the other two cases, particularly in that of yttrium, there had been marked absorption.

In addition measurements and observations were made at intervals of the growth and conditions of the plants, the points noted being the length of the green leaves, length and condition of the rootlets, and the condition of the florets and the flower spike. These observations and measurements will be found set out in the accompanying Table (p. 353).

While the results obtained were not in all cases concordant, a few definite points may be made. In the first place, all the bulbs in the experimental solutions reached maturity before the controls in water.

Secondly, the plants in the cerium carbonate solution were the first to attain maturity, though their development was not so marked as that of those in the lanthanum carbonate, which followed next in point of time.

The effect of the yttrium solution was somewhat anomalous, since while all the plants matured a few days before the controls, the rootlets were dwarfed, and looked yellow and unhealthy from the beginning.

The lanthanum ion seems to have a special effect on the flower stalks, resulting in very tall plants. No irregularities manifested themselves in the inflorescences.

Histological investigation of growing root-tips under the influence of the above reagents.

The varying effects produced by the different metallic ions are best seen from the accompanying photomicrographs (Plate I). Speaking generally it may be stated that the cerium and lanthanum ions have a decidedly stimulating effect on cell-division in the rootlets, while that of yttrium has a deleterious effect.

The following are brief notes of the histological examination of slides prepared from each of the twelve plants as mentioned in the Table. They were stained by Heidenhain's iron-alum, haematoxylin method.

1. *Cerium carbonate.* (*Nos.* 2 *and* 3.) (*See photomicrograph* 2.)

In both these preparations a noticeable feature is the beautiful regularity of the arrangement of the cells, the sections showing a large number of

Measurement of roots and leaves in cm.

Started Dec. 16th	No.	Jan. 8, 23rd day Roots only	Feb. 8, 54th day Roots	Feb. 8 Leaves	Feb. 11, 57th day Roots	Feb. 11 Leaves	Feb. 15, 61st day Roots	Feb. 15 Leaves	Feb. 21, 70th day Roots	Feb. 21 Leaves	Feb. 28, 74th day Roots	Feb. 28 Leaves	Mar. 4, 78th day Roots	Mar. 4 Leaves
Cerium Carbonate 0·007%	1	1	1 Look rotten	4·5 Withered	No development	—	—	—	—	—	—	—	—	—
	2	1	1	7	1	7 All florets open	—	—	—	—	—	—	—	—
	3	7·5	12	12	15	15	15	17·5 All florets open	—	—	—	—	—	—
Lanthanum Carbonate 0·01%	4	12·5	15	10	15	17	15	17 All florets open	—	—	—	—	—	—
	5	7	7·5	10	8	12·5	8	15 Florets open	—	—	—	—	—	—
	6	4	4	8	5	10	5	12·5	5	12·5 Florets open	—	—	—	—
Yttrium Carbonate 0·017%	7	2·5	4	7·5	4	10	6	12·5 Florets open	—	—	—	—	—	—
	8	2	2	7·5	2	12·5	2	12·5	2	12·5	2	15 Florets open	—	—
	9	2	2·5	6	3	7·5	3	7·5	3	7·5	3	8 Florets open	—	—
Controls Water	10	7	10	10	10	12·5	10	12·5	10	15	10	15	12	16 Florets open
	11	4	6	10	6	11·5	6	12	7·5	12·5	7·5	15	7·5	15 Florets open
	12	2	4·5	7·5	5	10	5	10	7·5	12·5	Withered	—	—	—

rather small, closely packed, square cells, containing many dividing nuclei. The cell-walls are clearly defined, and the cytoplasm is granular and stains well. In many cases the nuclei are elongated, and show one or two nucleolus-like chromatin dots surrounded by a clear space.

2. *Lanthanum carbonate.* (*Nos.* 4, 5 *and* 6.) (*See photomicrograph* 3.)

The same regularity of the arrangement of the cells is seen in these sections, but it is not quite as marked as in the cerium preparations. Many dividing nuclei are seen, and the nucleolus-like dots above referred to are also conspicuous. The cytoplasm is faintly granular, and the cell walls are less sharply defined than in Nos. 2 and 3 (cerium).

3. *Yttrium carbonate.* (*Nos.* 7, 8 *and* 9.) (*See photomicrograph* 4.)

In this series hardly any dividing cells are to be seen. The arrangement of the cells is irregular. The nuclei are very deeply stained, and irregular in size and shape. The cell walls are not distinct, and the cytoplasm is scanty, ill-defined, and very faintly staining. The whole appearance is that of an irregular mass of cells, with scattered deeply-stained nuclei, and presents a very different picture from the compact and regular arrangement shown in the cerium and lanthanum series.

4. *Controls—tap-water.* (*Nos.* 10, 11 *and* 12.) (*See photomicrograph* 1.)

In these sections no great amount of cell-division is noticeable. The cells are fairly regularly arranged, and the nuclei are deeply stained. They are for the most part in the resting condition, and many exhibit the darkly staining dots resembling nucleoli, each surrounded by a clear space.

CONCLUSIONS.

1. Marked effects are produced upon the dividing cells of hyacinth rootlets by the addition of the carbonates of cerium, lanthanum and yttrium to the medium. The concentration of these substances necessary to produce physiological effects is very small.

2. The cations produce diverse effects; lanthanum especially, and cerium being favourable to growth and cell-division, while yttrium is unfavourable.

3. The lanthanum ion has a special effect on the flower stalk, causing an increase in length.
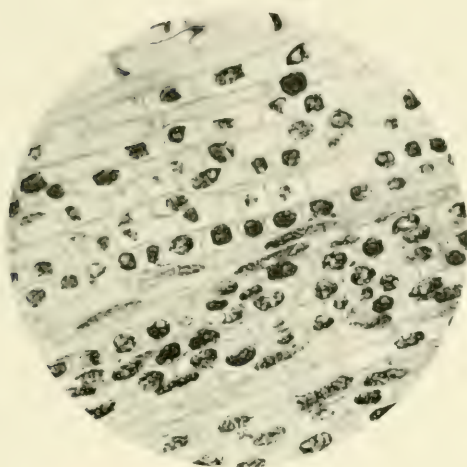
Fig. 1.

Control tapwater.   × 460 diameters.
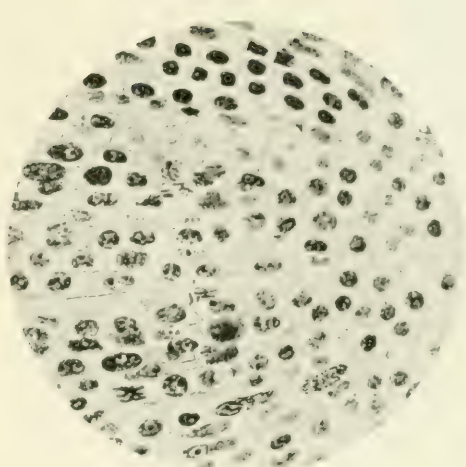
Fig. 2.

Cerium carbonate ·007 %.   460 diameters.
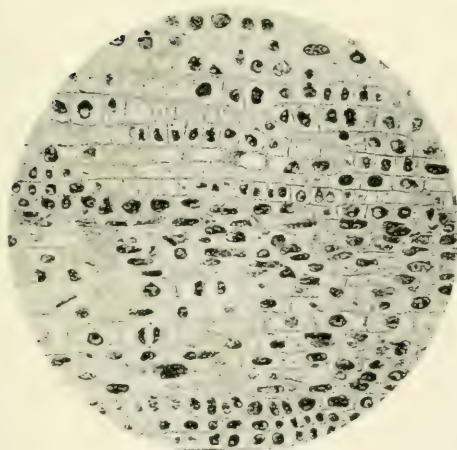
Fig. 3.

Lanthanum carbonate 0·01 %.   < 460 diameters.
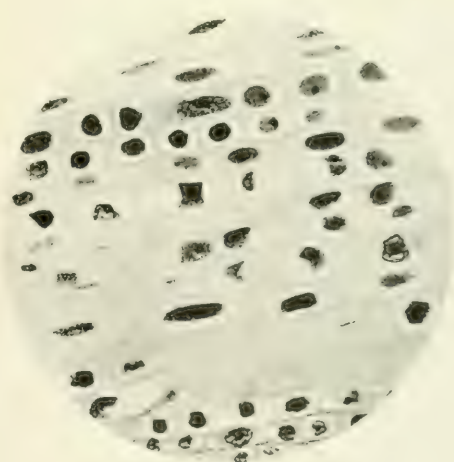
Fig. 4.

Yttrium carbonate ·017 "...   160 diameters.

4. The cytological effects are best seen in the accompanying photographs. In the cerium and lanthanum preparations there is a marked increase in cell-division, accompanied by a beautiful regularity in the arrangement of the cells. With yttrium there is a diminution in cell-division, and the cells are irregularly arranged.

I should like to take this opportunity of thanking Professor Benjamin Moore for suggesting the line of research, and for his kindly help and criticism throughout the work.

REFERENCES.

Coppin (1912), *Biochem. J.* **6**, 416.
Moore, Knowles and Roaf (1908), *Biochem. J.* **3**, 279.
Moore, Roaf and Whitley (1905), *Proc. Roy. Soc.* B. **77**, 103.
Ransom (1912), *Biochem. J.* **6**, 151.
Whitley (1905), *Proc. Roy. Soc.* B. **77**, 137.

XXXV. ON THE CHEMICAL NATURE OF SUBSTANCES FROM ALCOHOLIC EXTRACTS OF VARIOUS FOODSTUFFS WHICH GIVE A COLOUR REACTION WITH PHOSPHOTUNGSTIC AND PHOSPHOMOLYBDIC ACIDS. (PRELIMINARY COMMUNICATION.)

BY CASIMIR FUNK, *Beit Memorial Research Fellow*, AND ARCHIBALD BRUCE MACALLUM, *Beit Memorial Research Fellow*.

From the Biochemical Department, Lister Institute.

Two colour reactions have recently been described, one with phosphotungstic acid for uric acid [Folin and Macallum, 1912], the other with phosphotungstic and phosphomolybdic acids for polyphenols [Folin and Denis, 1912], which they recommend for the determination of these substances. One of us previously observed during the fractionation of yeast and rice-polishings [Funk, 1913] that the vitamine-fraction constantly gave a blue coloration with both reagents, that the reaction gradually disappeared during the further purification of the products and at the same time that the curative power for polyneuritis in birds slightly diminished. As these reacting substances might be of some importance for the process of curing, we have tested a large number of compounds with both reagents, in order to obtain some information as to the chemical nature of these substances, and we have found that a certain number of them which occur in nature show the colour reactions.

With the uric acid reagent certain purine bodies and some tyrosine derivatives give a very strong reaction. The alcoholic extracts of different foodstuffs, especially those which are known to be rich in vitamines, give in general both reactions very markedly. The substances giving the reaction seem to pass into the phosphotungstic acid filtrate and we are proceeding to isolate them.

The reactions with purine and pyrimidine derivatives.

Substance				Uric acid reagent	Phenol reagent
Xanthine	...	...	...	−	+ +
Hypoxanthine	...	...	...	−	+
Paraxanthine	...	...	...	−	−
Guanine	...	...	...	−	+ +
Alloxantin	...	...	...	+ + +	+ + +
Alloxan	...	...	...	−	−
Thymine	...	...	...	−	−
Theophylline	...	...	...	−	−
3-Methyl-uric acid .. ·	...	...	+	+ +
7- ,, ,,	...	...	...	trace	+ +
Hydantoin	...	...	...	−	−
Uracil ...	...	...	...	−	−
Guanidine carbonate	...	...	−	−
Hydantoic acid	...	...	...	−	−
Allantoin	...	...	...	−	−
Adenine	...	...	...	−	−
Uridine	...	...	...	−	−
Guanosine	...	...	...	−	trace
Adenosine	...	...	...	−	−
Cytidine nitrate	...	...	...	−	−
Yeast nucleic acid ...	...	...	trace	trace
Thymonucleic acid	...	...	−	−

Tyrosine derivatives.

l-Tyrosine	...	...	...	−	+ + +
Nitro-tyrosine	...	...	...	−	−
3-4-Dihydroxyphenylalanine	...	...	+ + +	+ + +
2-Aminotyrosine	...	...	...	+ + +	+ + +
3-Aminotyrosine	...	...	...	+ +	+ + +
l-Tyrosine anhydride	...	...	−	− ?
Glycyl-*l*-tyrosine	...	...	...	trace	+ +

We have also investigated a number of amino-acids, polypeptides and diketopiperazines, not including tryptophane, oxy-tryptophane and oxy-proline, all of which were entirely negative to both reagents.

Foodstuffs.

Ceridin (alcoholic extract of yeast)	...	trace	+
Zymin ...	...	...	...	trace	+
Alcoholic extract of rice-polishings	...	+	+ +
Subs. $C_{21}H_{19}O_9N_5$ from vitamine-fraction of yeast	...	...	...	−	−
Subst. $C_{20}H_{23}O_9N_5$ from the same fraction	−	+
Nicotinic acid from yeast and rice	...	−	..
$C_{26}H_{20}O_9N_4$ from vitamine-fraction of rice	−	..
Alcoholic extract from caseinogen (crude)	+	+
Alcoholic extract of milk	...	...	+	+
Whey powder from milk	...	...	+	+
Filtrate from milk precipitated by acid	...	trace	+
Alcoholic extract of whey powder	...	trace	+
Cod liver oil and the aqueous extract	...	+	+ +
Alcoholic and aqueous extract of Rous' chicken sarcoma	...	...	trace	+ +

We see from the table that the reactions are very specific for purine derivatives and polyphenols and they may therefore serve as a guide as to what groups the substances giving the reactions may belong. As the reactions are very sensitive, it seems to us that they might be used to ascertain the purity of phosphatides and other substances like caseinogen which are prepared from foodstuffs, and for which up to the present we possess no standard of purity. This test has already been found very useful in the investigation of the vitamine-fraction.

The table for purine derivatives shows that a substitution of one hydrogen atom in the purine ring lessens or destroys the power to give the uric acid reaction. In the case of the phenol reagent this is also brought about when two hydrogen atoms are substituted. The colour reactions with tyrosine derivatives and alloxantin were remarkably stable as compared with the others.

REFERENCES.

Folin and Macallum junr. (1912), *J. Biol. Chem.* **11**, 265; **13**, 363.
Folin and Denis (1912), *J. Biol. Chem.* **12**, 239.
Casimir Funk (1913), *Brit. Med. J.* April 12th; *J. Physiol.* **46**, 173.

XXXVI. THE PRODUCTION OF ACETALDEHYDE DURING THE ANAEROBIC FERMENTATION OF GLUCOSE BY BACILLUS COLI COMMUNIS (ESCHERICH).

By EGERTON CHARLES GREY, 1851 *Exhibition Scholar.*

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Acetaldehyde has long been known as a product of alcoholic fermentation by yeast [see Roeser, 1893]. It has likewise been found to be formed by the leaves of higher plants when their metabolism is restricted to anaerobic conditions [Kostytschew, 1913].

In view of this wide distribution, it was to be expected that acetaldehyde would be a product of bacterial fermentation. This is the case.

The quantity of acetaldehyde found by Roeser in various wine musts after a fermentation lasting from five to fifteen days, varied from 20–200 milligrams per litre of the fermented fluid. In the case of *B. coli communis* acting on glucose, one litre of fluid containing originally 15 grams of the sugar yielded in 15 days when half the sugar had been fermented 2·34 mg. of acetaldehyde, while, at the same time, 1·375 grams of alcohol had been produced. Since in the case of the true alcoholic fermentation quoted above, the quantity of alcohol formed was certainly twenty times that produced in the experiment with *B. coli communis,* it will be seen that the ratio of acetaldehyde to alcohol is of the same order in the two cases.

It is not at present settled, in the case of yeast fermentation, whether acetaldehyde is a primary product or whether it results from the oxidation of preformed alcohol, the following result is therefore significant in showing that the production of acetaldehyde by *B. coli communis* is related to the formation of alcohol, carbon dioxide, and acetic acid, rather than to the other products.

The experiment consisted in comparing the production of acetaldehyde from normal *B. coli communis* (Escherich) with that from a strain derived from the normal organism by selection with sodium chloracetate [see Penfold, 1911, and Harden and Penfold, 1912]. This derived strain had completely

lost the power of producing gas from carbohydrates. That it was closely related to the original organism was demonstrated by testing the agglutinating power of both the original and the derived organism towards a rabbit serum obtained after inoculating with the former. Both organisms agglutinated completely with the serum up to a dilution of one part of the serum in 25,600 of normal saline solution.

REACTIONS EMPLOYED FOR THE DETECTION AND DETERMINATION OF ACETALDEHYDE.

(1) *Rimini's reaction* [1898].

A few drops of diethylamine are added to a similar quantity of a solution of sodium nitroprusside. A blue colour appears which rapidly fades (2–3 minutes). If this solution be now diluted with 1–2 c.c. of water a pale yellow or yellow-green solution is obtained but if diluted with 1–2 c.c. of a solution containing acetaldehyde a blue colour results characteristic of this aldehyde. Though specific for acetaldehyde the reagent is not so delicate as that of Schiff, and the coloration is moreover less permanent.

(2) *Schiff's reagent*. This reagent was used for the determination of acetaldehyde.

It is important to make sure that no alkali is present in the fluid to be tested by Schiff's reagent since even the alkalinity of tap water produces a definite coloration. The maximum intensity of colour, by simple addition of alkali, was obtained with 0·1 c.c. normal KHO, added to 50 c.c. distilled water containing 0·25 c.c. Schiff's reagent. A greater concentration of alkali destroys the colour, which is regained on the addition of acid. The solution before examination was therefore always tested with phenolphthalein paper.

Acetone is said to give a coloration with Schiff's reagent, but this effect which is not produced for many hours cannot possibly be confused with the reaction for acetaldehyde in which case the coloration reaches a maximum in about twenty minutes and has very considerably faded in less than an hour. It is important to emphasise this fact, since Mendel [1911] states that he found acetone amongst the products of the action of *B. coli communis* and certain other organisms on glucose.

DETAILS OF THE DETERMINATION OF ACETALDEHYDE.

(1) About 750 c.c. of the fluid containing the liquid and solid products of fermentation (acetaldehyde, alcohol, and calcium salts of volatile and non-volatile acids etc.) was treated with oxalic acid in excess of that necessary for

the complete precipitation of the calcium. When the precipitate of calcium oxalate had settled, as much as possible of the supernatant liquor was removed with the aid of a siphon, and distilled. The first 400–500 c.c. of the distillate was made alkaline with barium hydroxide and redistilled, using a fractionating column, until 100 c.c. had been collected. In both distillations the receivers were well cooled by means of ice.

50 c.c. of this solution were used for the determination of the acetaldehyde, the method of procedure being an adaptation of that of Ryffel [1909].

(2) A standard solution of formaldehyde was prepared as described by Ryffel. The strength of this solution was determined by comparing the colour produced from it on the addition of Schiff's reagent, with that produced on adding the same quantity of the reagent to a solution of acetaldehyde prepared by the distillation of a known weight of lactic acid.

0·02494 gram lactic acid was converted into acetaldehyde. The intensity of colour produced on adding 0·5 c.c. Schiff's reagent to 100 c.c. of this acetaldehyde solution was 1·9 times that of the standard formaldehyde colour as measured by a Duboscq tintometer, 100 c.c. of the standard formaldehyde solution was therefore equivalent to $\frac{0·02494}{1·9} = 0·01312$ gram lactic acid.

Applying the ratio determined by Ryffel empirically for these conditions, viz.: 0·4 mg. formaldehyde = 3·435 mg. lactic acid = 1·765 mg. acetaldehyde, it follows that 100 c.c. of standard formaldehyde solution contains $\frac{0·4 \times 0·01312}{3·435} = 1·528$ mg. formaldehyde, and is equivalent to $\frac{1·528 \times 1·765}{0·4} = 6·471$ mg. acetaldehyde.

(3) 50 c.c. of the solution of formaldehyde described in paragraph (2) was diluted fivefold and compared with the distillate described paragraph (1), with the result:

The ratio of $\frac{\text{standard}}{5 \times \text{distillate}} = \frac{1}{1·33}$.

From this it was calculated that the total amount of acetaldehyde in the original litre of fermented fluid was 2·34 mg.

DISCUSSION OF RESULTS.

In order to demonstrate the relationship between the production of acet aldehyde and that of alcohol, carbon dioxide, hydrogen and acetic acid, the analyses of the products formed by the normal and the artificially selected strain are compared below, the results being calculated to 100 grams of the sugar.

	Products from glucose	
	Normal B. coli communis per cent.	Strain artificially selected by the chloracetate method per cent.
Hydrogen	0·42	Nil
Carbon dioxide	16·9	Nil
Alcohol	18·1	5·3
Acetic acid	18·5	10·8
Formic acid .	9·7	11·1
Lactic acid	36·8	68·0
Succinic acid	0·7	0·8
Acetaldehyde	31·345 mg.	Nil
⎰ Glucose decomposed	7·465 gram	7·755
⎱ Acetaldehyde found	2·34 mg.	Nil

It will be seen that the artificially selected organism has (as in the case of the organism examined by Harden and Penfold), besides having lost the power of producing gas, also produced less alcohol and acetic acid. Coincidentally with these changes there has been an abolition or great reduction of the yield of acetaldehyde.

In the light of these results we may consider the question of the origin of the acetaldehyde.

If this substance were a secondary product formed by the oxidation of alcohol through the activity of oxidases concerned in the cell-growth as was suggested by Roeser for the case of yeast, then since there is a large excess of alcohol in both cases, the amount of acetaldehyde formed in the two experiments should be approximately the same, or at least, of the same order. Moreover in view of the fact that not only was air excluded from the fermentation flasks, but that in the experiment in which acetaldehyde was produced, 350 c.c. of hydrogen had been evolved, such an oxidation would seem unlikely to have occurred. The strongly reducing conditions of the experiment may be well seen from the experiment in which Harden attempted to employ asparagine as a source of nitrogen. This substance, he found, was completely reduced to ammonium succinate with corresponding diminution in the hydrogen evolved.

It may be suggested therefore that the production of acetaldehyde and part of the alcohol occur simultaneously. This would agree with the view held by Kostytschew [1912] that acetaldehyde is an intermediate product in alcoholic fermentation and would moreover suggest that part of the alcohol produced by the action of B. coli communis on glucose, passes through at least one of the same stages as that produced by the zymase of yeast.

From a consideration of the structure of glucose and mannitol, and the

fact that *B. coli communis* produces about twice as much alcohol from the latter as from the former, Harden [1901] suggested that the terminal group $- CH(OH) . CH_2OH$ which occurs twice in mannitol though only once in glucose, is related to the production of alcohol.

Mannitol $CH_2OH \cdot CHOH \cdot (CHOH)_2 \cdot CHOH \cdot CH_2OH.$
Glucose $CH_2OH \cdot CHOH \cdot (CHOH)_2 \cdot CHOH \cdot CHO.$
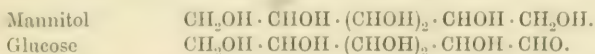
If then the final group $- CHOH . CH_2OH$ conditions the production of alcohol, the corresponding group in glucose $- CHOH . CHO$ might stand in the same relation to acetaldehyde.

Clearly since an accumulation of this product would be harmful to the organism natural selection would have evolved the organism capable of transforming the aldehyde by reduction into ethyl alcohol, or oxidation to acetic acid.

Further results will be shortly forthcoming with regard to the production of acetaldehyde from other substances allied to glucose.

SUMMARY.

(1) Acetaldehyde has been detected as a product of the action of *B. coli communis* on glucose, under anaerobic conditions.

(2) By artificial selection of *B. coli communis* by means of growth on sodium chloracetate, strains of the original organism have been obtained which produce either a greatly diminished amount of acetaldehyde or none at all.

(3) It has been found that the production of acetaldehyde is related to the formation of alcohol, carbon dioxide and hydrogen rather than to the other products. This has been ascertained by a comparison of the products formed by normal *B. coli communis* with those from an artificially selected strain produced by growth on agar containing sodium chloracetate.

(4) It is therefore suggested that acetaldehyde is a primary and not a secondary product of fermentation, and also that the process of alcohol formation by *B. coli communis* is in part analogous to the alcoholic fermentation set up by the zymase of yeast and to processes which occur in the leaves of higher plants.

REFERENCES.

Harden (1901), *J. Chem. Soc.* **79**, 610.
—— and Penfold (1912), *Proc. Roy. Soc. B.* **85**, 415.
Kostytschew (1912), *Zeitsch. physiol. Chem.* **79**, 130.
—— (1913), *Zeitsch. physiol. Chem.* **83**, 105.
Mendel (1911), *Centr. Bakt. Par.* II. **29**, 290.
Penfold (1911), *Proc. Roy. Soc. Med.* 97.
Rimini (1898), *Chem. Zentr.* **2**, 277.
Roeser (1893), *Ann. Inst. Pasteur.* **7**, 41.
Ryffel (1909), *J. Physiol.* **39**. Proc. v.

XXXVII. THE BIOCHEMICAL SYNTHESIS OF THE FATTY ACIDS.

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The methods by which fat is formed in the living organism remain at present completely unknown to us. Even the chemical reactions by which fatty acids are built up, comparatively simple though these must be, have hitherto found no satisfactory explanation. The one fact which appears to be completely established, by a large mass of experimental evidence, is that the carbohydrate of the food may be converted into fat inside the living organism, although neither the place where this change takes place nor the method by which it is accomplished is known with any degree of certainty.

The evidence as to the formation of fat from protein is less convincing, but it is at any rate possible that a conversion of protein into fat may also take place.

But little may be learnt from an attempt to correlate the composition of the fat stored up in the organism with the nature of the food supplied. There is abundant evidence that fatty acids taken in the food may be merely stored up unchanged in the body. The glycerides of palmitic, stearic and oleic acids are the constituents which most generally occur. Acids belonging to more highly unsaturated series than oleic acid have been demonstrated but these are more probably connected with further changes in the building up of fat into complex molecules, possibly of the nature of lecithin, than with the synthesis of the fatty acids themselves.

Two of the most prolific factories of fat are perhaps to be found (1) in plants, in such nuts as that of the cocoa-nut tree (*Cocos Nucifera*), where an abundant transformation of carbohydrate into fat must take place, and (2) in animals in the active mammary gland.

In both these instances, where a comparatively rapid conversion of

carbohydrate into fat is probably taking place the resultant fats are characterised by the presence of considerable quantities of the lower fatty acids. In cocoa-nut oil, the acids containing the even numbers of carbon atoms from six to eighteen, in butter from four to twenty, have been described. In these acids the carbon atoms are linked in straight chains and there is no evidence that any acid with a branched structure exists.

The question now arises whether the normal fatty acids present in butter are products of synthesis or of degradation. Knoop [1904] and Dakin [1908, 1909] have shown that the fatty acids are broken down by oxidation of the β-carbon atom; all the lower fatty acids present in butter may therefore be derived by oxidation from the arachidic or stearic acids present.

Some evidence on this point may be obtained from agricultural experiments; the problem has been directly investigated in an attempt to determine the reason of the variations which occur in the proportion of volatile fatty acids present in butter fat. Swaving (1906) carried out feeding experiments in the North of Holland to determine the cause of the low percentage of volatile soluble acids. Van der Zande and Siegfeld showed that a diet rich in carbohydrate, e.g. turnips, increased the proportion of the lower fatty acids and more recently Siegfeld [1907] and Amberger [1907] have shown that the increase is more especially in the insoluble volatile acids (i.e. caprylic, capric and lauric). Amberger, in a series of experiments carried out on the same set of cows showed that whereas food rich in protein such as malt germs diminishes the proportion of lower fatty acids, food rich in carbohydrate such as turnips increases this proportion. If the percentage of the lower fatty acids increases with the amount of the carbohydrate in the food, it would appear more probable that they exist as intermediate synthetic products on their way to the higher fatty acids, than as degradation products. Such evidence as exists is therefore in favour of a synthesis in which all fatty acids containing even numbers of carbon atoms from four to twenty linked together in straight chains are formed from carbohydrate in some way through the agency of the mammary gland.

Previous Hypotheses as to the Nature of the Reactions by which Fatty Acids are formed from Carbohydrate in the Animal Organism.

Emil Fischer suggested that stearic and oleic acids are formed by the condensation of three hexose molecules or of six triose (glycerose) molecules in such a way that a straight chain containing 18 carbon atoms

is formed. From this, by further processes of oxidation and reduction, stearic and oleic acids are formed. Palmitic acid with its chain of 16 carbon atoms would be compounded from two pentose and one hexose molecules. Glucose, gluconic and glucuronic acids are suggested as the precursors of the pentose molecules. In favour of this hypothesis it is difficult to find any evidence either of a chemical or biological nature. Against it the following considerations may be urged:

(a) No laboratory method is known by which two hexose molecules may be made to condense in such a way that a straight chain of twelve carbon atoms is produced.

(b) Pentoses are known to exist in the organism in combination in the nucleoproteins but there is no indication that the pentoses are in any way connected with the formation of fat or with normal carbohydrate metabolism.

(c) If it be granted that the fatty acids of butter are products formed synthetically from carbohydrate, the hypothesis presents insuperable difficulties. No combination of hexose and pentose molecules will produce myristic acid ($C_{14}H_{28}O_2$) by direct addition, yet this acid occurs commonly in fats, e.g. lard, butter, cod-liver oil and many vegetable fats. The existence of an intermediate tetrose sugar would have to be assumed as a normal constituent.

3 hexose molecules	give stearic acid.
2 pentose and one hexose molecules	,, palmitic acid.
2 pentose and one tetrose ,,	,, myristic acid.
2 hexose molecules	,, lauric acid.
2 pentose ,,	,, capric acid.
2 tetrose ,,	,, caprylic acid.

There is no evidence of the formation of a tetrose molecule in the organism and it is exceedingly unlikely that a regular series of fatty acids should be formed in this way.

(d) In the case of stearic acid the reduction of seventeen hydroxyl groups would be assumed.

This hypothesis has therefore no evidence in its favour and involves reactions which are not analogous with any of those known to us. It does not therefore furnish us with a satisfactory explanation of the problem under consideration.

The second hypothesis, which is perhaps the more generally accepted, is that the fatty acids are built up by repeated condensations of a compound containing two carbon atoms. This was first suggested by Nencki and afterwards developed by Magnus-Levy [1902], Leathes and others who regarded acetaldehyde as the substance from which by a series of aldol condensations

the straight chains containing even numbers of carbon atoms were formed.
The reactions involved would be represented by the following equations:

(1) $CH_3 . CHO + CH_3 . CHO = CH_3 . CHOH . CH_2 . CHO$
(2) $CH_3 . CHOH . CH_2 . CHO = CH_3 . CH_2 . CH_2 . COOH + H_2O$
$+ \dfrac{H-}{H} \longrightarrow O.$
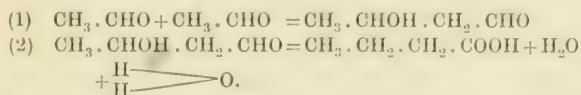
If the aldol on the other hand were reduced to butyl aldehyde, it would
be available again to take part in a similar condensation:

$CH_3 . CHOH . CH_2 . CHO + H_2 = CH_3 . CH_2 . CH_2 . CHO + H_2O$
$CH_3 . CH_2 . CH_2 . CHO + CH_3 . CHO = CH_3 . CH_2 . CH_2 . CHOH . CH_2 . CHO.$
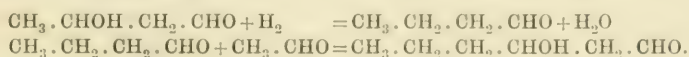
a normal six-carbon-atom chain being thus produced.

In favour of this hypothesis, it may be urged:

(a)   It does account for the production only of those fatty acids containing even numbers of carbon atoms, since only multiples of two will exist.

(b)   Hoppe-Seyler showed that by the action of caustic alkali on lactic acid at from 200°–300°, acetic, butyric and caproic acids were formed. Pasteur had previously shown that butyric and caproic acids were formed by the bacterial fermentation of sugar. Acetic aldehyde may be obtained from lactic acid and may therefore be a degradation product of sugar.

On the other hand it is open to the following criticisms:

(a)   Lieben [1883, 1901] and his pupils have shown that when the higher aldehydes condense with acetaldehyde under the influence of dilute alkalies, the resulting aldehydes possess a branched and not an open-chain structure:

$$CH_3 . CH_2 . CH_2 . CHO + CHO . CH_3 \longrightarrow \begin{array}{l} CH_3 . CH_2 . CH . CHO \\ \qquad\qquad | \\ \qquad\qquad CHOH \\ \qquad\qquad | \\ \qquad\qquad CH_3. \end{array}$$
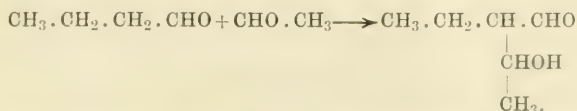
It has since been shown that both aldol and crotonaldehyde will undergo auto-condensation with the formation of a normal eight-carbon-atom chain [Raper, 1907; Smedley, 1911]; but the difficulty of adding on acetic aldehyde to a higher aldehyde so as to build up chains increasing by the addition of two carbon atoms has not been surmounted. One must therefore assume that the condensation of aldehydes in the body does not take place in the same manner as it does when brought about by the action of condensing agents in the laboratory.

(b)   No free aldehydes other than the sugars have been detected in the body. If present in quantity they would probably be injurious to the life of the cell. Parnas [1910] has shown that an enzyme is present in the liver by which free aldehydes are at once removed.

(*c*). There is no biological evidence that acetaldehyde is formed as an intermediate substance in the body metabolism.

The aldol condensation does not therefore furnish us with a satisfactory analogy for the method by which the fatty acids are built up.

A survey of the general methods of producing fatty acids in the laboratory shows that the most satisfactory method by which fatty acids may be built up by increments of two carbon atoms is by means of Reformatski's reaction in which aldehydes are condensed with bromoacetic ester in the presence of zinc;

$$R.CHO + BrCH_2.COOEt + Zn + H_2O = R.CHOH.CH_2.COOEt + Zn\genfrac{}{}{0pt}{}{OH}{Br}.$$
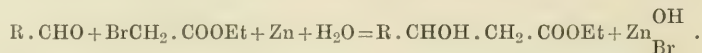
As however neither zinc nor bromoacetic ester occurs in the body, this does not furnish us with any helpful analogy for biochemical synthesis.

The Degradation-products of Carbohydrates: their suitability as Units in the biochemical synthesis of Fatty Acids.

But little is known as to the manner in which carbohydrate breaks down within the body. It has been repeatedly established that when a solution of glucose in Ringer's fluid is perfused through the isolated heart sugar disappears [Locke and Rosenheim, 1907; MacLean and Smedley, 1913]. This is the only instance in which it has been established beyond the region of controversy that sugar disappears when subjected to the action of an isolated organ. But even here the decomposition products of the sugar molecules are unknown. The controversy as to whether glycogen is a storage product or a stage in the normal metabolism of sugar throws little light on the problem under consideration. The discussion as to whether glucose is the source of the lactic acid in the animal organism has more bearing on the subject of fat formation. Embden has shown that the transfusion of blood rich in sugar through a glycogen-free liver resulted in the abundant formation of lactic acid: blood poor in sugar similarly transfused gave rise to lactic acid in inconsiderable amount. The formation of lactic acid from carbohydrate is also indicated by the experiments of Mandel and Lusk on phlorizin diabetes. It seems probable that both carbohydrate and protein may give rise to lactic acid in the body. The occurrence of lactic acid as a possible cleavage product of carbohydrate suggests that the breaking down of sugar takes place in such a way as to give rise to compounds containing three carbon atoms. Lactic acid itself is not a very reactive substance nor does

it appear a hopeful starting material for the synthesis of fatty acids. It is however closely related to pyruvic acid.

$$CH_3.CHOH.COOH \qquad\qquad CH_3.CO.COOH$$
$$\text{Lactic Acid} \qquad\qquad\qquad \text{Pyruvic Acid}$$
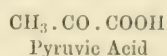
There is also evidence that pyruvic acid itself is probably of considerable importance in animal metabolism. It has been demonstrated that a close connection exists in the organism between the a-amino- and the a-keto-acids. Embden and Schmitz [1910, 1912] have shown that if a solution of ammonium pyruvate be perfused through a liver, alanine is formed. Fellner [1912] further showed that if a liver rich in glycogen be perfused with blood containing ammonia, alanine is formed, and from a consideration of Embden's experiments pyruvic acid is indicated as the intermediate substance. Neubauer and Knoop and Kertess [1911] have also suggested the formation of alanine from pyruvic acid in the body.

Knoop [1910] and Knoop and Kertess [1911] have shown that if γ-phenyl-α-amino-butyric acid be fed to a dog, a considerable proportion of the acid appears in the urine as the acetyl derivative; the same phenomenon was observed by Neubauer and Warburg [1910] in their perfusion experiments. There is some reason to believe that the acetylating agent may be pyruvic acid, since de Jong [1900, 1904] showed that ammonium carbonate and pyruvic acid react with formation of acetyl-alanine. It seems therefore probable that pyruvic acid may be an intermediate substance formed in the body from carbohydrate.

Pyruvic acid is a reactive substance, readily losing carbon dioxide under the influence of oxidising agents and forming acetic acid. A study therefore of its chemical properties and of its powers of condensation seemed of especial interest.

The condensation of Pyruvic Acid with Fatty Aldehydes and the oxidation of the products formed.

It had already been shown that if anhydrous hydrochloric acid be passed into a mixture of benzaldehyde and pyruvic acid, cinnamoyl-formic acid results [Erlenmeyer, 1901];

$$C_6H_5.CHO + CH_3.CO.COOH = C_6H_5.CH:CH.CO.COOH + H_2O.$$
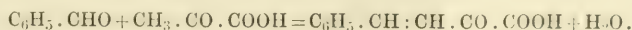
Later both benzaldehyde and cinnamyl aldehyde were condensed with pyruvic acid by adding a small amount of 10% caustic soda to the mixture [Erlenmeyer, 1903].

In order to make use as far as possible only of reagents which may be considered to bring about reactions somewhat similar to those brought about

by enzymes within the body, the condensation of the fatty aldehydes with pyruvic acid was attempted in very dilute alkaline solution. The intermediate unsaturated α-keto acid which was expected to result was not isolated, but the product was at once oxidised by silver oxide in alkaline solution or by hydrogen peroxide in neutral solution.

The behaviour of crotonaldehyde was first investigated.

<div align="center">EXPERIMENTAL.</div>

Condensation of crotonaldehyde with pyruvic acid and oxidation of the product formed.

5 grams pyruvic acid, 5 grams crotonaldehyde, 75 cc. n . NaOH and 1 litre of water were added together and left for three days at the room temperature, the solution being approximately 1/50 normal. The liquid became deep yellow but no insoluble oil separated as in the condensation of crotonaldehyde alone. The solution was neutralised by the addition of 12·5 cc. n . H_2SO_4 and steam distilled to remove any free aldehyde.

<div align="center">*Oxidation of Reaction-product.*</div>

Silver oxide was precipitated from 30 grams silver nitrate and added to the solution of the condensation product of crotonaldehyde with pyruvic acid after it had been steam distilled. 200 cc. of a 1/3 normal solution of baryta were gradually added and the whole allowed to stand over night. Next morning the silver oxide had been largely converted to silver. The precipitate was filtered off, washed and concentrated under reduced pressure until 50 cc. remained. In order to convert any hydroxy-acid that might conceivably be present to the corresponding unsaturated acid, 10 grams of baryta were added and the mixture boiled for 30 minutes.

Excess of sulphuric acid was then added and the whole steam-distilled. 1500 cc. of the steam distillate required 28·5 cc. normal potash for neutralisation. The neutral distillate was evaporated almost to dryness and to the potassium salt so obtained, 10 % H_2SO_4 was added. Crystals separated which melted at 132° after once recrystallising from dilute alcohol. The melting point was unchanged on mixing with a specimen of sorbic acid prepared by the condensation of crotonaldehyde and bromoacetic ester, and hydrolysis of the ester formed. The crystals were therefore satisfactorily identified as sorbic acid.

In subsequent experiments the oxidation of the neutral condensation product was carried out by means of hydrogen peroxide. An amount of hydrogen peroxide exactly equivalent to the pyruvic acid originally taken

was used and the neutral mixture of condensation product and peroxide left to stand over night at the ordinary temperature; the product was concentrated under reduced pressure and steam distilled as before, and the final product consisted of a mixture of acetic and sorbic acids, the yield being somewhat improved by this means. From 5 grams of crotonaldehyde 0·5 g. sorbic acid was thus obtained.

The reaction must therefore have proceeded as follows:

$$CH_3.CH:CH.CHO + CH_3.CO.COOH = CH_3.CH:CH.CH:CH.CO.COOH$$
$$CH_3.CH:CH.CH:CH.CO.COOH + H_2O_2 = CH_3.CH:CH.CH:CH.COOH + H_2O + CO_2$$
Sorbic Acid.
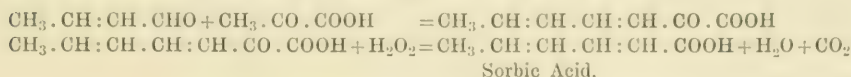
The condensation of Butyl Aldehyde with Pyruvic Acid.

10 grams of butyl aldehyde, 10 grams of pyruvic acid and 150 cc. normal potash were shaken up with 2 litres of water and at the end of 12 days the mixture was neutralised and concentrated under diminished pressure. To the concentrated residue silver oxide from 43 grams of silver nitrate and 200 cc. n/3 baryta were added. After standing over night, the silver precipitate was filtered off and the filtrate concentrated to 250 cc., 50 grams of baryta added and boiled for 30 minutes. The whole was then acidified with dilute sulphuric acid and distilled in steam. 1500 cc. of distillate were neutralised by 59·2 cc. normal potash and evaporated to dryness. The residue was acidified and extracted with ether. After evaporating off. the ether, the residue was distilled under a pressure of 20 mm. About 3 grams boiling from 130°–140° were obtained. The liquid rapidly decolourised bromine water and gave on analysis the numbers required for the compound $C_6H_{10}O_2$.

0·1409 g. ; 0·3262 g. CO_2; 0·1134 g. H_2O.
C 63·14 % H 8·94 %.
Calc. for $C_6H_{10}O_2$ C 63·15 % H 8·77 %.

In subsequent experiments, the product similarly prepared appeared to consist of a mixture of octylenic acid (probably obtained by the self-condensation of the butyl aldehyde) and of hexylenic acid obtained from butyl aldehyde and pyruvic acid. The difficulty of separating these in a small quantity of a liquid mixture is considerable.

In another experiment where hydrogen peroxide was used as the oxidising agent as described under crotonaldehyde, the product obtained distilled under reduced pressure (15 to 20 mm.) from 120°–128° and gave on analysis the following numbers.

0·1220 g. ; 0·2858 g. CO_2: 0·1074 g. H_2O.
C 63·85 %: H 9·75 %.

In investigating the condensation of iso-valeraldehyde and oenanthol with pyruvic acid, chiefly the products of condensation of the aldehydes with themselves were isolated. From the condensation product of iso-valer-aldehyde and pyruvic acid, a small amount of the barium salt of an acid was obtained, the percentage of barium in which agreed with that required for the barium salt of the corresponding unsaturated keto-acid.

Condensation of these higher fatty aldehydes with pyruvic acid under varying conditions is now being investigated. The condensation of croton and butyl aldehydes with pyruvic acid and the oxidation of the product formed with hydrogen peroxide in neutral solution furnishes a method by which an unsaturated fatty acid may be built up containing two more carbon atoms than the aldehyde from which it is derived. These condensations have also been investigated under similar conditions in the aromatic series [Smedley and Lubrzynska, 1913].

Conclusions.

The hypothesis now brought forward [Smedley, 1912] suggests that pyruvic acid, formed in the body as a decomposition product of carbohydrate, is the starting-point for the synthesis of the fatty acids. The stages which are assumed to occur are represented by the following equations:

(1) $CH_3 . CO . COOH = CH_3 . CHO + CO_2$.
     Pyruvic Acid → Acetaldehyde.

(2) $CH_3 . CHO + CH_3 . CO . COOH = CH_3 . CHOH . CH_2 . CO . COOH$
     $CH_3 . CH : CH . CO . COOH + H_2O$.
     Pentylenic α-keto-acid.

(3a) $CH_3 . CH : CH . CO . COOH + O = CH_3 . CH : CH . COOH + CO_2$.

(4a) $CH_3 . CH : CH . COOH + 2H = CH_3 . CH_2 . CH_2 . COOH$.
     Butyric Acid.

(3b) $CH_3 . CH : CH . CO . COOH = CH_3 . CH : CH . CHO + CO_2$.
     Pentylenic α-keto acid.

(4b) $CH_3 . CH : CH . CHO + CH_3 . CO . COOH = CH_3 . CH : CH . CHOH . CH_2 . CO . COOH$.
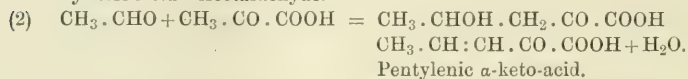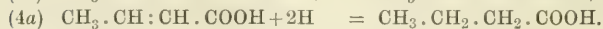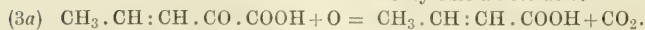
and by reactions similar to 3a and 4a
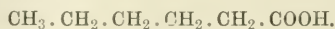
$$CH_3 . CH_2 . CH_2 . CH_2 . CH_2 . COOH.$$
Caproic acid.

The evidence supporting this hypothesis may be briefly summarised as follows.

1. Pyruvic acid is probably a degradation product of carbohydrate in the body.

The perfusion experiments of Embden, Knoop and Neubauer show that pyruvic acid is converted into alanine through the agency of the liver cells

and that a close connection exists between the α-amino- and α-keto-acids. Pyruvic acid may probably be an intermediate stage in the transformation from glycogen to alanine (Fellner).

There is some reason to believe that in the acetylation of certain amino-acids which has been observed both in perfusion and in feeding experiments, pyruvic acid is the acetylating agent.

The close connection between alanine and pyruvic acid suggests that the alanine group of the protein molecule may furnish an additional source of the pyruvic acid available for the synthesis of fatty acids.

2. The decomposition of pyruvic acid into acetaldehyde and carbonic acid, which constitutes the first stage of this process, has been shown by Neuberg to be readily brought about by an enzyme present in yeast, termed "carboxylase."

3. The present hypothesis postulates that free acetaldehyde is not liberated but that the decomposition of the keto-acid is in some way regulated by the pyruvic acid with which the "nascent" aldehyde combines.

The condensation of fatty aldehydes with pyruvic acid has now been shown to take place in the laboratory under the influence of dilute alkalis at ordinary temperature.

4. Oxidation of the α-keto acid according to the equation

$$R \cdot CO \cdot COOH + O = R \cdot COOH + CO_2$$
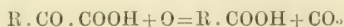
may be brought about in the laboratory by hydrogen peroxide at the ordinary temperature in neutral solution (p. 370).

5. The reduction of the unsaturated acid is the final stage; there is abundant evidence that reduction can take place in the body although very little is known as to the mechanism by which it is accomplished.

6. The α-keto-acid, synthesised as above, may be split into CO_2 and aldehyde, and a further condensation with pyruvic acid may then be effected. An acid with two more carbon atoms than the original aldehyde would thus be synthesised.

As yet no α-keto-acids have been detected within the body: it may be that they occur only within the cell and that reduction or oxidation always accompanies their liberation. The above hypothesis accounts for the formation of a series of straight chain acids beginning with four carbon atoms and increasing by increments of two carbon atoms: it involves only reactions which are analogous with those which are known to occur in the laboratory and there is reasonable evidence for believing that the starting material, pyruvic acid, can be formed from carbohydrate in the body.

REFERENCES.

Amberger (1907), *Zeitsch. Nahr. Genussm.* **13**, 516.
Dakin (1908), *J. Biol. Chem.* **4**, 63, 419 ; **5**, 173.
—— (1909), *J. Biol. Chem.* **5**, 303, 409 ; **6**, 203, 221, 235.
Embden and Schmitz (1910), *Biochem. Zeitsch.* **29**, 423.
—— (1912), *Biochem. Zeitsch.* **38**, 392.
Erlenmeyer (1901), *Ber.* **34**, 1817.
—— (1903), *Ber.* **36**, 2527.
Fellner (1912), *Biochem. Zeitsch.* **38**, 414.
De Jong (1900), *Rec. Trav. Chim.* **19**, 259.
—— (1904), *Rec. Trav. Chim.* **23**, 131.
Knoop (1904), "Der Abbau aromatischer Fettsäuren im Tierkörper."
—— (1910), *Zeitsch. physiol. Chem.* **67**, 489.
Knoop and Kertess (1911), *Zeitsch. physiol. Chem.* **71**, 252.
Lieben (1883), *Monatsh.* **4**, 10.
—— (1901), *Monatsh.* **22**, 289.
Locke and Rosenheim (1907), *J. Physiol.* **36**, 205.
MacLean and Smedley (1913), *J. Physiol.* **45**, 462.
Magnus-Levy (1902), *Beiträge*, **2**, 261.
Mandel and Lusk (1906), *Amer. J. Physiol.* **16**, 129.
Neubauer and Warburg (1910), *Zeitsch. physiol. Chem.* **70**, 1.
Neuberg and Hildesheimer (1911), *Biochem. Zeitsch.* **31**, 170.
Parnas (1910), *Biochem. Zeitsch.* **28**, 274.
Raper (1907), *J. Chem. Soc.* **91**, 1831.
Siegfeld (1907), *Zeitsch. Nahr. Genussm.* **13**, 616.
Smedley (1911), *J. Chem. Soc.* **99**, 1627.
—— (1912), *J. Physiol.* **45**, Proc. xxv.
Lubrzynska and Smedley (1913), *Biochem. J.* **7**, 375.
Swaving (1906), *Zeitsch. Nahr. Genussm.* **9**, 505.

XXXVIII. THE CONDENSATION OF AROMATIC ALDEHYDES WITH PYRUVIC ACID.

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In a preliminary communication published by one of us [Smedley, 1912], and in the foregoing paper [Smedley and Lubrzynska, 1913], the condensation of certain fatty aldehydes with pyruvic acid in very dilute alkaline solution has been described.

In order to obtain a better knowledge of the reaction and hence possibly to overcome some difficulties with which we found ourselves confronted in our experiments in the aliphatic series we have investigated the behaviour of certain aromatic aldehydes under similar conditions. A number of the $\beta\gamma$-unsaturated α-keto-acids have been prepared and converted by oxidation with hydrogen peroxide into the corresponding unsaturated acids containing one carbon atom less than the original keto-acid.

Claisen and Claparède [1882] studied the action of anhydrous hydrochloric acid on a mixture of benzaldehyde and pyruvic acid and obtained from the mixture cinnamoyl formic acid. This reaction was further studied by Erlenmeyer, junr. [1899, 1901], who showed that in addition to the above product γ-phenyl-β-benzylidene-α-ketobutyrolactone is formed:

$$C_6H_5 . CHO + CH_3 . CO . COOH = C_6H_5 . CH : CH . CO . COOH + H_2O.$$
$$2C_6H_5 . CHO + CH_3 . CO . COOH = C_6H_5 . CH : C . CO . CO . O . CH . C_6H_5 + 2H_2O.$$
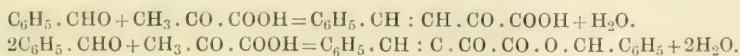
The condensations of benzaldehyde, cinnamic aldehyde, piperonal and anisic aldehyde with pyruvic acid have been studied in dilute alkaline solution. The reactions proceed in all cases readily at the laboratory temperature. The amount of potash used was generally such that the strength of the solution was from 1/40 to 1/10 normal, and the mixture was allowed to stand at the temperature of the laboratory for periods varying from 2 to 7 days. As the reaction proceeds the aldehyde disappears and the

liquid gradually becomes yellow. When the reaction is completed, the keto-acid is thrown down on acidifying the liquid, and after purification, is oxidised to the corresponding $\alpha\beta$-unsaturated acid.

Yellow crystalline products are formed by the condensation of anisic aldehyde and piperonal respectively with pyruvic acid, the formulae of which are shown on analysis to agree with those required for the β-unsaturated α-keto-acids:

$$R.CHO + CH_3.CO.COOH = R.CH:CH.CO.COOH + H_2O.$$
$$R.CH:CH.CO.COOH + H_2O_2 = R.CH:CH.COOH + CO_2 + H_2O.$$
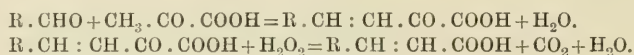
After we had completed the above series of experiments, we found that we had overlooked two papers by Erlenmeyer, junr. [1903, 1904], in which he had described the condensation of benzaldehyde and cinnamic aldehyde respectively with pyruvic acid in the presence of strong caustic soda (10 %). The products which we obtained on condensing cinnamic aldehyde with pyruvic acid were similar to those described by Erlenmeyer. He isolated two forms, an orange-red, melting at 75° and gradually converted *in vacuo* into a yellow form melting at 107°. Our products melted at 73° and 104° (uncorrected) respectively.

From anisic aldehyde and pyruvic acid yellow crystals melting at 130° were obtained. Piperonal under similar conditions gives beautiful yellow needles which at a temperature of about 70° undergo a remarkable transition into a deep orange-red form and finally melt at 163°. On keeping the red form so obtained in an evacuated desiccator or in a well-stoppered bottle it was still unchanged after a month had elapsed. If however it was exposed to the air it was gradually reconverted into the yellow variety.

On oxidation with the calculated quantity of hydrogen peroxide in neutral solution at the ordinary temperature the keto-acids split off carbon dioxide and form unsaturated acids:

$$R.CH:CH.CO.COOH + H_2O_2 = R.CH:CH.COOH + CO_2 + H_2O.$$
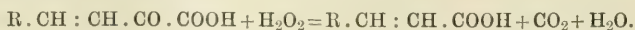
Silver oxide which was successfully used to oxidise the corresponding keto-acids in the fatty series [Smedley, 1912], was found to be without action in the aromatic series.

EXPERIMENTAL.

Condensation of piperonal with pyruvic acid. 4 g. of pyruvic acid and 6 g. of piperonal were shaken up with 1 litre of water and 140 cc. N. KOH added; the mixture was allowed to stand at the ordinary temperature for a week, during which it became yellow. The small amount of unchanged

piperonal was filtered off and the solution neutralised with 36 cc. N. H_2SO_4. The neutral solution was extracted with ether several times to free it from traces of unchanged aldehyde. Air was drawn through to remove the last traces of ether and the solution acidified. A yellow solid was precipitated and allowed to settle: it was then filtered off and well washed with cold water. After several recrystallisations from dilute alcohol it was obtained in beautiful yellow needles which turned to deep orange-red at about 70° and melted at 163°.

0·1062 g.; 0·2336 g. CO_2; 0·0368 g. H_2O.
C 59·98 %; H 3·85 %.
Calc. for $C_{11}H_8O_5$, C 60·00 %; H 3·63 %.

Condensation of anisic aldehyde with pyruvic acid. 5 g. anisic aldehyde and 3·5 g. pyruvic acid were shaken up with 1 litre of water and 70 cc. N. KOH added, the mixture being allowed to stand for 4 days during which a yellow colour developed. It was then neutralised with 25·05 cc. N. H_2SO_4 and the mixture treated exactly as in the case of the condensation of piperonal with pyruvic acid.

The yellow needles finally obtained melted at 130° and no change in colour was observed either on standing or on heating.

0·1100 g.; 0·2585 g. CO_2; 0·0500 g. H_2O.
C 64·09 %; H 5·00 %.
Calc. for $C_{11}H_{10}O_4$, C 64·07 %; H 4·85 %.

The specimen, part of which gave the above results on analysis, was allowed to remain in a closed test-tube for two weeks and then analysed :

0·0972 g.; 0·2330 g. CO_2; 0·0407 g. H_2O.
C 65·32 %; H 4·63 %.

A week later it was again analysed:

0·1208 g.; 0·2927 g. CO_2; 0·0562 g. H_2O.
C 65·98 %; H 5·16 %.

The acid was then recrystallised and again analysed :

0·1244 g.; 0·2972 g. CO_2; 0·0564 g. H_2O.
C 65·11 %; H 5·06 %.

The preparation was repeated and the freshly prepared product analysed :

0·1169 g.; 0·2743 g. CO_2; 0·0518 g. H_2O.
C 64·10 %; H 4·91 %.

The acid therefore on standing undergoes a slight decomposition, the nature of which was not ascertained.

Condensation of cinnamic aldehyde with pyruvic acid. 5 g. cinnamic aldehyde and 4 g. pyruvic acid were shaken up with 1 litre of water and 80 cc. of normal potash. The mixture was allowed to stand for 4 days at the

ordinary temperature, neutralised and treated as in the experiments already described with piperonal and anisic aldehyde.

The condensation product was obtained in two forms; red crystals melting at 73°, which pass gradually into a yellow substance melting at 104°. In one experiment red crystals melting at 73° were exposed in an evacuated desiccator for 48 hours: they had then become yellow and melted at 95°. After another 24 hours the melting point was 101° and after recrystallisation from dilute alcohol it rose to 103°. Analysis showed that the composition of the two forms after drying in a vacuum desiccator was identical.

$$0.1274 \text{ g.}; \quad 0.3344 \text{ g. } CO_2; \quad 0.0600 \text{ g. } H_2O.$$
$$C \ 71.58 \, \%; \quad H \ 5.17 \, \%.$$
$$\text{Calc. for } C_{12}H_{10}O_3, \quad C \ 71.28 \, \%; \quad H \ 4.95 \, \%.$$

Oxidation of dioxymethylene-benzylidene-pyruvic acid:

$$CH_2O_2C_6H_3CH : CH . CO . COOH.$$

The keto-acid was dissolved in alcohol, neutralised with potash and the theoretical quantity of hydrogen peroxide then added. It is important that no excess of the peroxide be present or the yield will be found to be diminished. The oxidation product was heated for an hour on a water-bath to complete the reaction and then acidified. A very pale yellow acid was precipitated, which after one recrystallisation was almost white and melted at 242°.

$$0.1074 \text{ g.}; \quad 0.2462 \text{ g. } CO_2; \quad 0.0423 \text{ g. } H_2O.$$
$$C \ 62.47 \, \%; \quad H \ 4.37 \, \%.$$
$$0.1148 \text{ g.}; \quad 0.2690 \text{ g. } CO_2; \quad 0.0458 \text{ g. } H_2O.$$
$$C \ 62.51 \, \%; \quad H \ 4.32 \, \%.$$
$$\text{Calc. for } C_{10}H_8O_4, \quad C \ 62.50 \, \%; \quad H \ 4.16 \, \%.$$

Oxidation of cinnamylidene-pyruvic acid:

$$C_6H_5 . CH : CH . CH : CH . CO . COOH.$$

The oxidation was carried out in a manner similar to that just described for the piperonal product. The acid obtained after recrystallisation from benzene and from alcohol melted at 165° and was therefore identical with the cinnamylidene-acetic acid prepared by Perkin from malonic acid and cinnamic aldehyde, melting at 165°.

$$0.0844 \text{ g.}; \quad 0.2335 \text{ g. } CO_2; \quad 0.0442 \text{ g. } H_2O.$$
$$C \ 75.47 \, \%; \quad H \ 5.80 \, \%.$$

After again recrystallising:

$$0.1093 \text{ g.}; \quad 0.3052 \text{ g. } CO_2; \quad 0.0578 \text{ g. } H_2O.$$
$$C \ 76.12 \, \%; \quad H \ 5.85 \, \%.$$
$$\text{Calc. for } C_{11}H_{10}O_2, \quad C \ 75.86 \, \%; \quad H \ 5.74 \, \%.$$

Oxidation of methoxy-benzylidene-pyruvic acid:

$$CH_3O . C_6H_4 . CH : CH . CO . COOH.$$
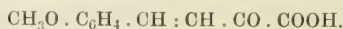
This acid was dissolved in alcohol, carefully neutralised with potash and hydrogen peroxide added. In this case it was found that the best yield was obtained on adding 1·5 times the theoretical quantity of the peroxide. After several recrystallisations, the acid melted at 172°. Analysis gave the following result:

$$0\cdot1020 \text{ g.}; \quad 0\cdot2517 \text{ g. } CO_2; \quad 0\cdot0530 \text{ g. } H_2O.$$
$$C\ 67\cdot23\ \%; \quad H\ 5\cdot70\ \%.$$
$$\text{Calc. for } C_{10}H_{10}O_3, \quad 67\cdot41\ \%; \quad H\ 5\cdot62\ \%.$$

Condensation of benzaldehyde with pyruvic acid. The condensation was carried out as in the cases already described, but the intermediate keto-acid was not isolated. The product was at once oxidised in neutral solution with hydrogen peroxide.

It was found in this case of the greatest importance to have the solution exactly neutral and to avoid any excess of the peroxide.

On acidification, cinnamic acid was precipitated. It was identified by taking the melting-point of a specimen mixed with pure Kahlbaum's cinnamic acid.

The yields in the above experiments varied from 50–70 % of the theoretical.

REFERENCES.

Claisen and Claparède (1882), *Ber.* **14**, 2472.
Erlenmeyer, junr. (1899), *Ber.* **32**, 1450.
—— (1901), *Ber.* **34**, 817.
—— (1903), *Ber.* **36**, 2527.
—— (1904), *Ber.* **37**, 1318.
Smedley (1912), *J. Physiol.* **45**, *Proc.* xxv.
—— and Lubrzynska (1913), *Biochem. J.* **7**, 364.

XXXIX. THE PRECIPITATION OF EGG-ALBUMIN BY AMMONIUM SULPHATE. A CONTRIBUTION TO THE THEORY OF THE "SALTING-OUT" OF PROTEINS.

By HARRIETTE CHICK and CHARLES JAMES MARTIN.

INTRODUCTION.

The first systematic and quantitative researches upon the precipitation of proteins by salts in large amounts were made by Hofmeister and his pupils.

In the experiments of Kauder [1886] and Lewith [1888] with serum proteins, and Hofmeister [1888] with egg-protein, comparison was made of the precipitating power of a large series of electrolytes, as a result of which the latter were arranged in what is known as the Hofmeister series. Most of the experiments were made with sodium salts, among which sulphate, phosphate, acetate, citrate, tartrate, chromate, chloride, nitrate and chlorate formed a series arranged in descending power of precipitation; of the kations examined lithium was the most effective, and sodium, potassium, ammonium and magnesium came afterwards in order of decreasing efficiency.

Hofmeister [1889] came to the conclusion that the precipitation was caused by the electrolytes depriving the protein of the amount of water necessary to keep it in solution, and was confirmed in this view by the results of some experiments showing the influence of various salts in modifying the imbibition of water by gelatin [1891]; work in this direction was later extended by Pauli [1898] and Pauli and Rona [1902].

Spiro [1904] demonstrated that the precipitation from their solutions of caseinogen and gelatin by sodium sulphate was analogous to the "salting out" of alcohol recently studied in detail by de Bruyn [1900]. In neither case is the phenomenon one of simple precipitation, since, owing to the appropriation of water by the salt, separation into two phases occurs. Each phase contains all the constituents of the system and any alteration in one of

the three constituents leads to readjustment of the composition and relative volumes of the two phases. Spiro also pointed out that, since in the case of alcohol the effect of electrolytes is not attributable to the constituent ions, any influence of the latter in the salting out of proteins must be regarded as a subsidiary phenomenon.

Spiro's conception explains to some extent the divergent results obtained in the precipitation of proteins by the addition of neutral salts, when the whole conditions are not maintained constant.

The series of observations which we are about to record concern the "salting out" by ammonium sulphate of pure recrystallised egg-albumin. Our observations show that in this case also we have to deal with the separation of the original system (itself not homogeneous) into two distinct phases, and that the influence upon the volume of these phases of concentration of protein, salt and water in the system is, as Spiro found for caseinogen and gelatin, analogous to what occurs in alcohol, salt and water mixtures. In addition, however, we find that the charge carried by the protein particles is an important factor in the final equilibrium.

The results of the experiments will first be set forth and the proposed explanation discussed later.

PRECIPITATION OF PURE EGG-ALBUMIN BY AMMONIUM SULPHATE.

Material. The material employed was egg-albumin crystallised from egg-white in presence of ammonium sulphate according to the method of Hopkins and Pinkus [1898]. The albumin was recrystallised once or twice, separated from the mother liquor by pressing between filter paper, and finally dissolved in distilled water. A concentrated stock solution was thus obtained, the composition of which, as regards (1) protein, (2) ammonium sulphate, (3) water was accurately ascertained by analysis, and which, when diluted to a suitable degree, served for most of the following experiments.

Since the salt employed for "salting out" in these experiments was also ammonium sulphate, the small concentration of the latter always present in the original albumin solution presented no complication; an allowance was made for this amount in the calculations. In those cases where an electrolyte-free solution was required, the albumin solution was previously dialysed.

Egg-albumin prepared in this way we believe to be as homogeneous a protein as it is possible to obtain. Hopkins [1899–1900] came to the conclusion that egg-albumin, crystallised from faintly acid ammonium sulphate solutions by the above method, was a pure substance. The

rotatory power remained absolutely constant after repeated recrystallisations (p. 312) and the proportion of carbon, hydrogen, nitrogen and sulphur, as well as the ash, remained constant. His experiments were made with four different samples after three or four recrystallisations.

1.   *Influence of concentration of salt upon the amount of protein precipitated.*

Mellanby [1907] made a quantitative study of the influence of concentration of ammonium sulphate on the precipitation of the proteins from horse serum, but, as far as we are aware, no experiments have as yet been made with a pure protein. In the present instance two sets of experiments were made, in both of which the concentration of protein was about 1 %. In the first the concentration of protein in the whole system was left constant $= 1.11$ % by weight, and the ratio salt to water was varied (Table I and Fig. 1). In the second set the ratio protein to water was kept constant and the precipitation studied by varying the amount of salt present; the concentration of protein in the whole system varied from 1.0 % to 0.93 % (by weight). (Table II and Fig. 2.)

TABLE I.

Precipitation of pure egg-albumin with ammonium sulphate; influence of concentration of salt.

Temperature, 20°.
Protein constant $= 1.11$ % by weight of total system.
Ratio salt/water varying.

Albumin g.	Water g.	Salt g.	G. albumin in 100 g. total system	G. salt in 100 g. total system	G. albumin in 100 g. filtrate
1·00	69·00	20·00	1·11	22·22	1·089
1·00	68·00	21·00	,,	23·33	0·711
1·00	67·00	22·00	,,	24·44	0·302
1·00	66·00	23·00	,,	25·55	0·104
1·00	65·00	24·00	,,	26·66	0·0315
0·50	32·35	12·15	,,	27·00	trace
0·50	32·00	12·50	,,	27·77	trace

The method of experiment was as follows. Mixtures were prepared by weighing into stoppered bottles the required amount of water and protein, and the necessary amount of ammonium sulphate was then added in large crystals and gently shaken. This prevented over-saturation with ammonium sulphate in the neighbourhood of the crystals, the large size of which

prevented a too rapid solution. The bottles were placed in a thermostat at
20°, for from 1 to 2 hours, the contents filtered and the protein estimated in
the filtrate by weighing the coagulum formed on heating.
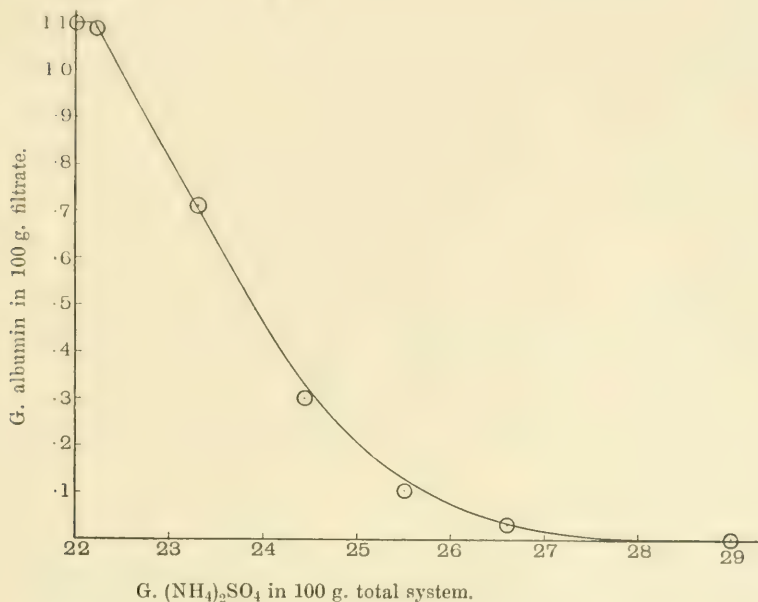
Fig. 1. Influence of concentration of the salt upon precipitation of egg-albumin by $(NH_4)_2SO_4$
at 20°, see Table I; protein constant$=1\cdot11$ % (by weight) of total system, ratio salt to
water varying.

TABLE II.

*Precipitation of pure egg-albumin with ammonium sulphate;
influence of concentration of salt.*

Temperature, 20°.
Ratio protein/water constant$=1\cdot3/100$.

Albumin g.	Water g.	Salt g.	G. salt to 1·3 g. protein and 100 g. H_2O	G. albumin in 100 g. filtrate
1·30	100	29	29	0·998
1·3	100	29·7	29·7	no precipitation.
1·30	100	30	30	0·938
1·95	150	46·5	31	0·733
1·3	100	32	32	0·487
2·6	200	66	33	0·273
2·6	200	67	33·5	0·232
2·6	200	70·01	35·0	0·105
2·6	200	76	38·0	0·022
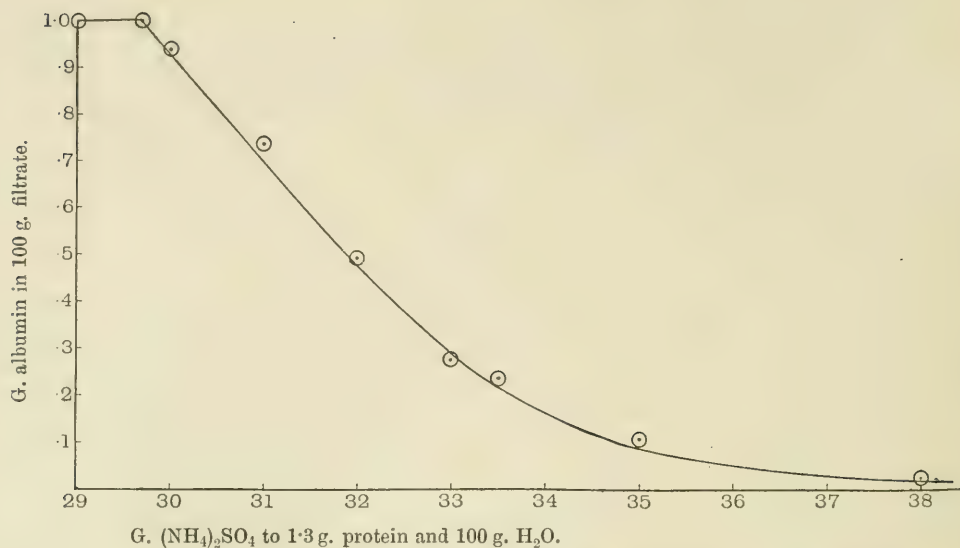
Fig. 2. Influence of concentration of the salt upon the precipitation of egg-albumin by $(NH_4)_2SO_4$ at 20°, see Table II; ratio protein to water constant=1·3 to 100, amount of salt present varying.

The curves in Figs. 1 and 2 are both of the same general form, in both cases the percentages of albumin in the filtrate are plotted as ordinates and the abscissae are respectively percentage by weight of ammonium sulphate in the whole system and grams of ammonium sulphate present, which explains the fact that the curve is steeper in the former instance. The point of commencing precipitation which is very sharply marked is at about the same concentration of salt in both cases, viz. 22·2 and 22·7 % ammonium sulphate respectively; the curve then descends steeply and approaches the base line asymptotically.

2. *Influence of concentration of protein.*

Kauder [1886] showed that serum albumin was more readily precipitated by ammonium sulphate if in more concentrated solution and determined the diminishing concentration of ammonium sulphate necessary to cause commencing precipitation in a series of solutions of increasing protein concentration. Hofmeister in 1888 published the results of similar experiments, using egg-white and potassium acetate and ammonium sulphate. Similar evidence has since been brought forward by other workers, e.g. Mellanby [1907], but in no case was a pure protein employed.

We have made an experiment with pure egg-albumin, estimating the

protein precipitated by a constant concentration of ammonium sulphate (ratio salt to water constant) when the amount of protein was varied. The results are given in Table III and graphically set forth in Fig. 3, where the proportion of the protein separated is plotted as ordinate against the concentration (percentage by weight of whole system) of protein in the original mixture as abscissa. Not only is more protein separated from the more concentrated

TABLE III.

Precipitation of pure egg-albumin with ammonium sulphate;
influence of concentration of protein.

Ratio salt/water constant = 31/100.
Concentration of protein varying.

Albumin g.	Water g.	Salt g.	G. albumin in 100 g. total mixture	G. albumin to 31 g. salt and 100 g. H$_2$O	G. albumin in 100 g. filtrate	G. albumin precipitated from 100 g. total mixture	Protein ppted. %
1·90	56·92	17·65	2·481	3·33	1·130	1·351	54·4
1·90	36·92	11·44	3·775	5·14	1·115	2·660	70·4
3·79	50·85	15·76	5·383	7·45	1·159	4·224	78·5
7·59	53·69	16·64	9·738	14·13	0·935	8·803	90·4
4·74	17·31	5·36	17·306	27·4	0·772	16·534	95·5
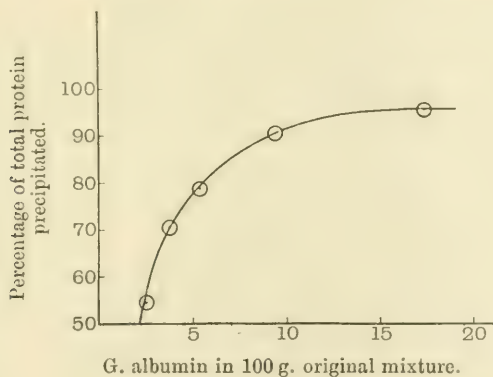
Fig. 3. Influence of concentration of protein upon the precipitation of egg-albumin by (NH$_4$)$_2$SO$_4$ at 20° C., see Table III; ratio salt to water constant = 31 to 100, amount of protein varying.

solution at a given concentration of salt, *but a greater proportion is precipitated* (see last column, Table III). The concentration of protein in the filtrate is not constant, but varies from 1·13 % to 0·77 % (col. 6, Table III), as the initial protein concentration is varied from 2·5 to 17·3 % (col. 4). This suggests that the precipitation is a phase separation, analogous to the case of "salting out" of alcohol with ammonium sulphate. As will be

seen later, when the results are given of determinations of the protein-salt-water content of filtrate and precipitate respectively, this was proved to be the case.

3. *Influence of hydrogen ion concentration.*

It is common experience, e.g. with serum, that the addition of a little acid enhances the amount of protein precipitated by the same concentration of ammonium sulphate, and that proteins not precipitated by saturation with sodium chloride are thrown down on acidification of the solution.

Mellanby [1907] called attention to the increased amount of precipitation of horse serum by neutral salts after addition of various acids, and gave some quantitative data, using sodium chloride. In the present investigation the influence of acidity was directly measured in a series of mixtures in which the concentration of protein and ammonium sulphate was maintained constant, and so chosen that precipitation had just begun in the control solution. The reaction of the solution was adjusted to various degrees of hydrogen ion concentration by the addition of small quantities of standard sulphuric acid.

Determinations of hydrogen ion concentration were made with the type of concentration cell described by Michaelis and Rona [1909], the contact fluid between the two cells being saturated potassium chloride solution. It is possible that the determinations of H^+ concentration are not very accurate owing to the high concentration of ammonium sulphate present; those in any one series are, however, perfectly comparable.

TABLE IV.

*Precipitation of pure egg-albumin with ammonium sulphate;
influence of hydrogen ion concentration.*

Experiment I at 18°.
G. ammonium sulphate in 100 cc. original mixture = 30·4.
G. protein in 100 cc. original mixture = 0·575.

G. protein present in 100 cc. filtrate	Hydrogen ion concentration in filtrate, in terms of normality
0·572	$10^{-5·27}$ (54 × 10^{-7})
0·391	$10^{-5·00}$ (101 × ,,)
0·097	$10^{-4·64}$ (231 × ,,)
0·062	$10^{-4·53}$ (296 × ,,)
0·048	$10^{-4·31}$ (492 × ,,)
0·035	$10^{-4·11}$ (780 × ,,)
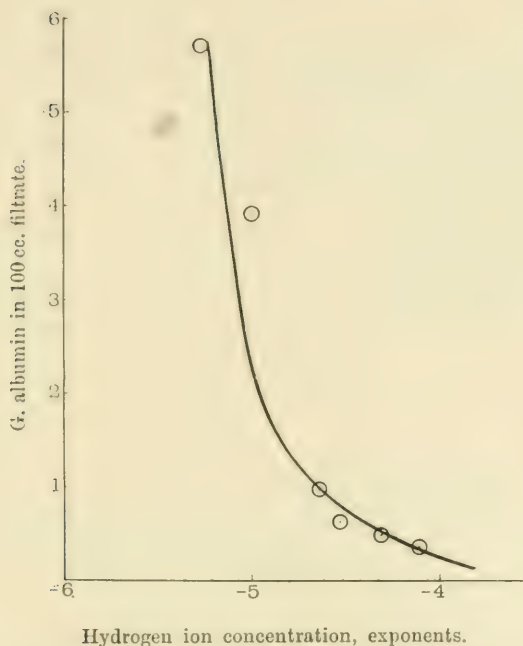
Fig. 4. Influence of hydrogen ion concentration upon the precipitation of egg-albumin by (NH₄)₂SO₄ at 18° (see Table IV).

Concentration of $(NH_4)_2SO_4$ in whole system, constant $= 30\cdot4$ grams per 100 cc.
        ,,   ,, Protein   ,,   ,,   ,, $= 0\cdot575$,,   ,,

The concentration of protein varied from 0·6 to 0·9 % and that of ammonium sulphate from 30·4 to 28·6 % (by volume) in the three different series of experiments set forth in Tables IV to VI. The mixtures were placed for two hours at 18° in order that equilibrium might be attained; they were then filtered and the concentration of protein and of hydrogen ions in the filtrate was determined.

The influence of hydrogen ion concentration was found to be very marked although the range through which it operates is not extensive. In one case (Exp. I, Table IV, protein concentration = 0·57 %, ammonium sulphate = 30·4 %) an increase in acidity from 54×10^{-7} normal (control) to 780×10^{-7} normal was enough to cause precipitation of nearly all the protein. In Exp. II, Table V (0·86 % protein and 30·03 % ammonium sulphate) and Exp. III, Table VI (0·9 % protein and 28·6 % ammonium sulphate) the effective range of hydrogen ion concentration was from 2·9 to 360×10^{-7} normal, and from 12 to 203×10^{-7} normal respectively.

TABLE V.

Precipitation of pure egg-albumin with ammonium sulphate;
influence of hydrogen ion concentration.

Experiment II at 18°.
G. ammonium sulphate in 100 g. original mixture = 30·03.
G. protein in 100 g. original mixture = 0·856.

No. of cc. N/10 H_2SO_4 (or equivalent) added in total volume of 27 cc.	No. of cc. N/10 NH_4OH (or equivalent) added in total volume of 27 cc.	G. protein in 100 cc. filtrate	Hydrogen ion concentration (filtrate), in terms of normality
—	1·0	0·864	$10^{-6.54}$ (2·88 × 10^{-7})
—	0·5	0·809	$10^{-6.01}$ (9·82 × ,,)
—	0·3	0·795	$10^{-5.72}$ (19·0 × ,,)
—	—	0·514	$10^{-5.40}$ (39·8 × ,,)
0·5	—	0·056	$10^{-4.98}$ (104 × ,,)
1·0	—	0·01 (about)	$10^{-4.45}$ (358 × ,,)

The last trace of protein present, however, does not appear to be precipitated by alteration of reaction alone, a slight trace in Exp. III being still left in solution at a hydrogen ion concentration of about 1/100 normal or 73000×10^{-7} normal.

The range of reaction where hydrogen ion concentration has its great effect is presumably just on the acid side of the iso-electric point (see below p. 392). From Exp. III it is seen that for ammonium sulphate change in hydrogen ion concentration at and on the alkaline side of the neutral point is without much influence (viz. from $H^+ = 12 \times 10^{-7}$ normal to 0.008×10^{-7} normal), see Table VI.

TABLE VI.

Precipitation of pure egg-albumin with ammonium sulphate;
influence of reaction, hydrogen ion concentration.

Experiment III at 18°.
G. $(NH_4)_2SO_4$ in 100 g. original mixture = 28·6.
G. protein ,, ,, ,, = 0·910.

Cc. N/10 H_2SO_4 (or equivalent) added in total volume of 25·4 cc.	Cc. N/10 $(NH_4)OH$ (or equivalent) added in total volume of 25·4 cc.	G. protein in 100 cc. filtrate	Hydrogen ion concentration (filtrate) in terms of normality
—	3·6	No precipitation	$10^{-9.11}$ (0·008 × 10^{-7})
—	2·0	0·899	$10^{-7.23}$ (0·59 ,,)
—	1·0	0·908	$10^{-6.56}$ (2·7 ,,)
—	—	0·899	$10^{-6.08}$ (12·0 ,,)
0·25	—	0·533	$10^{-5.45}$ (35 ,,)
0·5	—	0·174	$10^{-5.01}$ (97·5 ,,)
1·0	—	0·063	$10^{-4.69}$ (203 ,,)
5·0	—	trace	$10^{-2.45}$ (35000 ,,)
10·0	—	slight trace	$10^{-2.14}$ (73000 ,,)
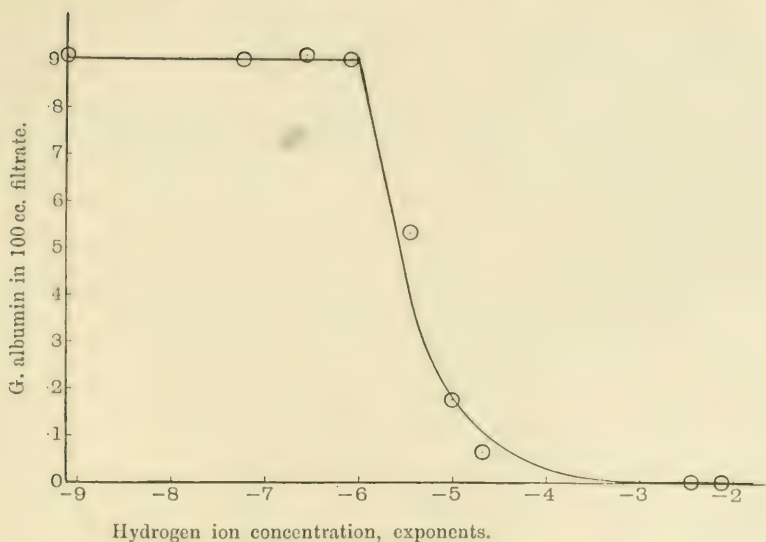
Fig. 5. Influence of hydrogen ion concentration upon the precipitation of egg-albumin by $(NH_4)_2SO_4$ at 18° (see Table VI).

Concentration of $(NH_4)_2SO_4$ in whole system, constant $= 28.6$ grams in 100 cc.

,, ,, protein ,, ,, ,, $= 0.910$,, ,,

H^+ concentration expressed as exponents.

4. *Influence of temperature.*

Lewith [1888] in case of ox-serum proteins, showed that rise in temperature assisted the precipitation by ammonium sulphate and Hofmeister [1888] made the same observation with egg-white and various salts. Spiro [1904] stated the same to be true of crystalline serum albumin and ammonium sulphate.

We have confirmed the above observations for serum proteins[1] and pure egg-albumin if the reaction be alkaline, but in faintly acid solution (10^{-5} normal) we have found the reverse to be true above 9°.

In Table VII and Fig. 6 are set forth the results of an experiment with 0.85 % protein and 28 % ammonium sulphate. A series of exactly similar solutions were placed for from 1 to 2 hours in a thermostat at the required temperature, after which they were rapidly filtered and the protein estimated in the filtrate. From 0° to 9° the temperature coefficient of precipitation was positive, above 9° it remained negative to 50°[2]. This can be readily

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[1] Mellanby [1907, p. 294], on the other hand, states that the temperature coefficient of serum-protein precipitation with $(NH_4)_2SO_4$ is negative between the temperatures of 0° and 40° C. and so small as to be unimportant.

[2] No denaturation occurred.

demonstrated if ammonium sulphate be added to an egg-albumin solution at 9° to the point of opalescence, and just short of the formation of a definite precipitate. If, then, the mixture be divided into three portions, of which one is placed at 0° and a second at 20°, a definite precipitation will occur at both temperatures, whereas the portion maintained at 9° will remain merely opalescent.

TABLE VII.

Influence of temperature upon the precipitation of pure crystalline egg-albumin with ammonium sulphate (28 %).

Protein content = 0·85 %.
Hydrogen ion concentration about 10^{-5} normal.

Temperature	Concentration of protein in filtrate, %
50	0·828
36	0·703
20	0·364
9	0·268
0	0·318
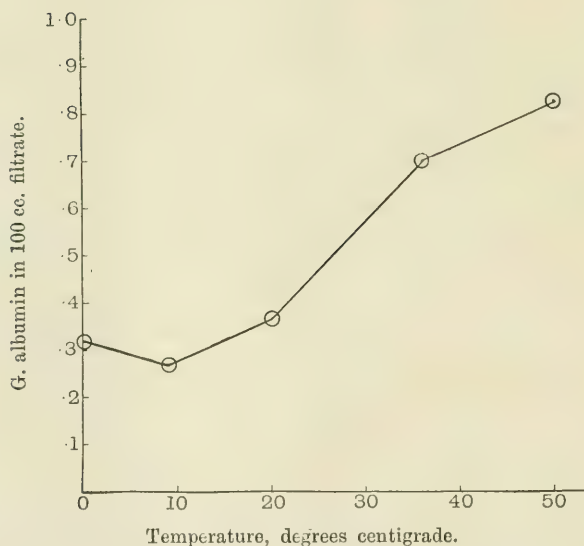
Fig. 6. Influence of temperature upon the precipitation of egg-albumin by $(NH_4)_2SO_4$, see Table VII.

Concentration of protein, salt, water and hydrogen ions in whole system constant.

INTERPRETATION OF RESULTS.

From the experiments detailed above, it is clear that the amount of protein precipitated from solution (volume of the protein-rich phase) is

dependent upon the amount of salt present, concentration of protein, concentration of hydrogen ions and temperature.

Spiro [1904] showed that, in the "salting out" of caseinogen and gelatin by sodium sulphate, the ratio of salt to water in the more protein-rich phase was less than in the watery phase—a similar relation to that found by de Bruyn [1900] in alcohol separation.

The same is true in the case of egg-albumin. An albumin solution was precipitated by ammonium sulphate and a complete analysis made of the original solution, the precipitate and the filtrate. In order to free the precipitate from adherent mother-liquor, it was not simply dried between filter paper, as done by Spiro, but placed between filter paper, surrounded by kieselguhr and submitted to a pressure of about 3 tons to the square inch. This pressure was, however, more than enough to squeeze out adherent mother-liquor, and actually removed some of the imbibed water of the protein-rich phase, for the concentration of salt in the liquid expressed, in the one case where it was collected and analysed, was only about half of that in the watery phase. The analyses given in Table VIII, where the results of these experiments are set out in detail, do not therefore express the composition of the two phases which were in equilibrium. The experiments prove, however, that the protein had appropriated some of the water for, notwithstanding this squeezing out of weak salt solution, the ratio salt to water in the compressed cake was considerably less than in the watery phase. See Table VIII.

This means that egg-albumin, like caseinogen and gelatin, dissolves or imbibes water just as alcohol does, and a mixture of protein, salt, water can be made to separate into two phases, either by the further addition of salt or of protein in the same way as a mixture of alcohol, salt, water separates on the addition of either salt or alcohol. In either case it amounts to increasing the concentration of salt in the watery phase.

To explain the influence of hydrogen ion concentration in the precipitation of egg-albumin, we must suppose that the electrical condition of the colloidal particles is a factor which modifies the ease with which they aggregate under the influence of the salt present. Hofmeister [1889] was influenced by a similar idea when he made parallel experiments with colloidal ferric hydroxide and egg-white, and Posternak [1901, 1 and 2], when investigating the precipitation of vegetable globulins, found that the efficiency of various electrolytes was influenced by the reaction of the suspension and presumably by the charge originally carried by the protein particles.

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TABLE VIII.

Precipitation of pure egg-albumin by ammonium sulphate;
composition of filtrate, pressed precipitate and press-liquor.

Exp.	Composition % by weight of	In original mixture	In pressed precipitate	In filtrate	In press liquor
I	Egg-albumin ...	9·56	64·65	0·84	—
	$(NH_4)_2SO_4$	23·35	29·83	26·67	—
	H_2O	67·08	6·39	72·49	—
	Salt present in 100 parts salt and water	25·82	17·6	26·9	—
II	Egg-albumin ...	12·48	63·69	0·59	—
	$(NH_4)_2SO_4$	22·60	29·91	27·04	—
	H_2O	64·92	8·47	72·37	—
	Salt present in 100 parts salt and water	25·82	22·1	27·2	—
III	Egg-albumin ...	9·47	73·57	1·58	0
	$(NH_4)_2SO_4$	24·09	22·04	27·66	15·2
	H_2O	66·42	6·39	70·76	84·8
	Salt present in 100 parts salt and water	26·62	22·47	28·1	15·2

The particles of proteins in acid or alkaline solutions carry respectively a positive or negative electric charge. Only at the iso-electric point, which is slightly to the acid side of the true neutral point in all cases hitherto investigated, does the charge disappear.

Salts like ammonium sulphate and sodium sulphate will cause separation into two phases, one protein-rich and the other protein-poor, whatever the charge upon the protein aggregates of the colloidal solution, if enough of the salt be added. Such phase separation is very materially assisted if the particles are positively charged, i.e. in solutions more acid than the iso-electric point. In the case of sodium chloride, indeed, acidification is necessary with many proteins.

From the analogy of the "salting out" of alcohols, phenols, etc. one must suppose that, by the gradual withdrawal of water from the protein aggregate by the salt, a critical dispersion point is reached when the surface tension at the interfaces causes the particles to run together. Supposing the particles are not iso-electric with the continuous phase, either from the original solution being acid or alkaline, or from the preferential adsorption of one of the ions of the electrolyte, the possession of charge will lower surface tension, so that, with charged particles, a higher concentration of salt will be required to arrive at the critical dispersion point. With negatively charged particles, in an alkaline solution, the charge cannot be neutralised by the more potent

ion SO_4'' which, if adsorbed, would still further increase the negative charge. We find that once the solution is more alkaline than the neutral point the amount of alkali added makes no difference[1] to the amount of salt required for precipitation, see Exp. III, Table VI and curve Fig. 5. The moment the reaction is made more acid than the iso-electric point and the protein particles carry a positive charge, this will at once be neutralised by the adsorption of SO_4'' ions. As these are in such high concentration they are apparently able to counteract in this way the maximum positive charge imposed upon the particles by the addition of acid (see Exp. III, Table VI, $H^+ = 0.007$ normal).

We venture to put forward the above interpretation from the analogous action of SO_4'' upon protein particles under conditions which permit of the demonstration of the existence of charge. In acid solution, protein particles, carrying a positive charge, have been shown to be sensitive to the anion of any electrolyte they may encounter. (Hardy [1900] for heated egg-white; Chick and Martin [1912] for denaturated serum proteins and egg-white; Chick [1913] for euglobulin and caseinogen.) Arguing from analogy we may suppose that in the case of egg-albumin the SO_4'' ion of ammonium sulphate will also be more readily adsorbed and any charge on the protein neutralised if the solution be originally on the acid side of the iso-electric point, i.e. if the protein particles carry a positive charge.

The statements detailed above have not actually been substantiated in case of pure egg-albumin, nor has the iso-electric point been determined. This has, however, been done in case of serum-albumin [Michaelis and Mostynski, 1910; Michaelis and Rona, 1910], and these two proteins otherwise display close similarity as regards the conditions of their solution or precipitation. We have not been able to put our interpretation to the direct test because it is impracticable to determine the charge carried by the particles of egg-albumin in the presence of excess of ammonium sulphate, nor under these conditions were we able to study the influence of addition of ions of varying valency. To test our hypothesis we therefore had

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[1] In the case of Na_2SO_4, which as regards the influence of acid behaves in an analogous manner to $(NH_4)_2SO_4$, excess of alkali (NaOH) favours precipitation, but, compared with the acid, a high concentration is required. In accordance with the explanation of the influence of reaction set forth above we presume that, while in acid solution the positively charged protein particles attract the SO_4 ion, in alkaline solution the Na ion of the sodium salt is preferentially absorbed. The electric charge on the particles is neutralised in both cases, but more readily in the first, owing to the greater potency of the SO_4 ion. With $(NH_4)_2SO_4$ no effect in solutions made alkaline with ammonia can be demonstrated, owing, presumably, to the low ionisation of $(NH_4)OH$, especially in the presence of excess of $(NH)_2SO_4$.

recourse to the expedient of withdrawing the water from the protein-water-combination by the addition of alcohol to the point where a surface tension is just manifest at the interfaces. In other words, the alcohol was added until the solution became opalescent but short of commencing precipitation, which could then be brought about by addition of a small amount of various electrolytes. Two sets of experiments were made; in one set the original protein solution was acid, and in the second set alkaline, and it was found, as was expected, that an electrolyte was efficient in causing precipitation of the protein in order of increasing valency of its anion in the first case and of its kation in the second. All solutions contained the same concentration of alcohol.

The results of a series of experiments with serum proteins are given in Table IX. In solution A (acid) salts containing divalent (ammonium sulphate) and trivalent (sodium citrate) anions caused precipitation in respective concentrations of 0·00055 and 0·00036 molar, whereas, in case of a monovalent anion (magnesium nitrate) about ten times the concentration was required. The valency of the kation was not without effect, but worked in the opposite direction; a small concentration of lanthanum nitrate (0·0007 molar) cleared up the original opalescence of the solution. For the same reason magnesium sulphate proved to be a much less efficient precipitant than ammonium sulphate.

An exactly converse set of results was obtained when the protein solution was originally made alkaline (B. Table IX). In this case lanthanum nitrate was the most powerful precipitant of all the salts tried and the sulphate of magnesium was much more effective than that of ammonium. At the same time a small concentration of sodium citrate (0·0007 molar) caused the solution to become clear.

With alkaline solutions higher concentration of lanthanum nitrate (0·0036 molar) prevented the formation of a precipitate, no doubt owing to the acquisition of a positive charge in excess of that needed to neutralise the negative one originally possessed. In acid solution an analogous result followed addition of sodium citrate to a concentration of 0·0007 molar.

Some experiments made with pure egg-albumin are set out in Table X. The original solution was acidified and after addition of sufficient alcohol to cause turbidity, determination was made of the concentration of a series of sodium salts necessary to cause a precipitate to form. The influence of increasing valency of the anion is very marked, sodium citrate and sulphate being respectively about 800 and 25 times as powerful in this respect as sodium chloride.

TABLE IX.

Precipitation of serum proteins by various electrolytes in presence of alcohol (almost to precipitation) at 0°.

Protein content=about 0·1 %.

A. In acid solution.

Salt	Conc. (molar)	Conc. (normal)	Degree of precipitation	Conc. (molar) required for complete precipitation
Na₃C̄it	0·00007	0·00022	⁻	0·00036
	0·00036	0·0011	+ +	
	0·00071	0·0022	+	
	0·0036	0·011	−	
(NH₄)₂SO₄	0·00011	0·00022	+ −	0·00055
	0·00055	0·0011	+ +	
	0·0055	0·011	+ +	
	0·055	0·11	+ +	
MgSO₄	0·00055	0·0011	+	0·0011
	0·0011	0·0022	+ +	
	0·0055	0·011	+ +	
	0·055	0·11	+ +	
Mg(NO₃)₂	0·0011	0·0022	+ −	0·0055
	0·0055	0·011	+ +	
La(NO₃)₃	0·00071	0·0022	−	
	0·0036	0·011	−	

B. In alkaline solution.

Salt	Conc. (molar)	Conc. (normal)	Degree of precipitation	Conc. (molar) required for complete precipitation
La(NO₃)₃	0·000071	0·00022	+ −	0·00036
	0·00036	0·0011	+ +	
	0·00071	0·0022	+ +	
	0·0036	0·011	+	
Mg(NO₃)₂	0·00011	0·00022	+ ·	0·00055
	0·00055	0·0011	+ +	
	0·0055	0·011	+ +	
	0·055	0·11	+ +	
MgSO₄	0·0011	0·0022	+ +	0·0011
	0·0055	0·011	+ +	
(NH₄)₂SO₄	0·0011	0·0022	+ − *	No precipitation
	0·0055	0·011	+ − *	
Na₃C̄it	0·00071	0·0022	−	,, ,,
	0·0036	0·011	−	

* Clearer than control solution containing alcohol only.

− =clear solution, clearer than the control, containing alcohol only.

− + =opalescent solution.

+ =partial precipitation.

+ + =complete precipitation, filtrate protein-free or containing trace only.

TABLE X.

Precipitation of pure egg-albumin, in acid solution, by sodium salts, in presence of alcohol almost to precipitation; influence of anions.

Protein content$= 0.7$ %.

Temperature, $0°$.

Salt	Concentration required to cause precipitation in presence of alcohol (Molar)	Concentration necessary to commence precipitation in absence of alcohol (Molar)
Citrate (neutral)	0·00013	1·37
Phosphate	0·00016	2·50
Tartrate	0·003	1·60*
Sulphate	0·004	1·69
Acetate	0·09	—
Chloride	0·10	4·3*
Chlorate	0·35	—
Chromate	0·4*	1·71*

* Did not precipitate.

In the same Table are given the results of a series of experiments in which precipitation took place in absence of alcohol and it will be seen that no such influence of valency can be detected here. The relation of the various electrolytes, with the exception of sodium citrate, is that expressed by the Hofmeister series [1888] and doubtless conditioned by their relative water-drawing capacity. When the protein is on the point of being precipitated after the necessary water-withdrawal has taken place, the influence of the anion or kation of the electrolyte present can complete precipitation by neutralising a charge. This occurs for example when protein almost precipitated by ammonium suphate is made slightly acid, or when to protein almost thrown out by alcohol a trace of an appropriate electrolyte is added. In the former case, as long as the solution remains alkaline, the effect of presence of ammonium sulphate with its divalent anion will rather be to increase the negative charge on the protein.

An additional piece of evidence in support of the view that the charge carried by the protein particles is an important factor in determining the concentration of salt requisite to occasion separation into two distinct phases, was obtained from studying the "salting out" of egg-albumin with calcium chloride. In this case the positive ion of the electrolyte is prepotent and we should expect it to act more efficiently if the solution containing the protein is made more alkaline than the iso-electric

point. This proved to be the case[1]. It was found that in a solution containing 0·8 % protein and 37·7 % CaCl, an opalescence was developed on standing for about 2 hours at 18° when the hydrogen ion concentration was about 23×10^{-7} normal. In presence of a small concentration of $Ca(OH)_2$, under otherwise similar conditions, the hydrogen ion concentration fell to about $0·05 \times 10^{-7}$ normal. In this case almost all the protein was precipitated and only a trace remained in the filtrate.

Precipitation by calcium chloride differs from that by ammonium sulphate in the fact that the process is irreversible, and the precipitate is formed slowly and becomes insoluble. We were not able to make satisfactory experiments with other electrolytes of the same character, e.g. the nitrates and chlorides of magnesium and barium, because with egg-albumin precipitation is only very partial even in alkaline solution, but in these cases also, we were able to satisfy ourselves that alkalinity of the solution assisted the separation of a precipitate (protein-rich phase).

SUMMARY.

1. The precipitation of egg-albumin by ammonium sulphate is, as Spiro demonstrated to be the case with sodium sulphate, and caseinogen and gelatin, due to the separation of the system into a protein-rich phase and a watery phase, and to a certain extent is analogous with the salting-out of alcohol.

2. The first effect of concentrated salt is to withdraw water from the protein aggregates. A surface tension is in consequence developed at the interfaces, which causes the protein particles to aggregate, thus dividing the system into two distinct phases (precipitate and filtrate).

3. All three constituents of the system, viz. protein, water and salt, are present in each phase; the proportion, however, is different. The precipitate (protein-rich phase) contains relatively little water and salt, and the filtrate (watery phase) relatively little protein. Under appropriate conditions practically all the protein may be precipitated, only a trace remaining in the filtrate.

The two phases are in equilibrium, and any alteration in the amount of any one of their three constituents is followed by a change both in their composition and volume. Thus increase in concentration of salt or of protein

[1] Precipitation by calcium chloride is also assisted in cases where the solution is markedly acid, a comparatively large concentration of acid (HCl) being needed to demonstrate the phenomenon. The explanation is exactly analogous to that offered for the case of sodium sulphate, see footnote, p. 393, but in the reverse sense, much more acid being required to produce this effect than is the case with alkali.

is followed by a corresponding increase in the protein-rich phase (precipitate). Owing to rigidity of the latter readjustment is, however, slowly accomplished.

4. The relative volume of the two phases (amount of precipitate) is altered by varying the temperature.

5. The exact proportion of salt, protein and water at which phase separation (precipitation) occurs and the relative volume of the two phases (amount of the precipitate) is very sensitive to hydrogen ion concentration in the neighbourhood of the iso-electric point. In the case of $(NH_4)_2SO_4$, when the hydrogen ion concentration varied from 10^{-6} to 10^{-5} normal, the amount of the precipitate increased from a negligible amount to a maximum.

6. With $CaCl_2$, in which the kation is prepotent, a similar effect was observed, but in the opposite direction.

7. An interpretation of the dominating influence of hydrogen ion concentration over this range is put forward and some experiments in support of it adduced.

The principles discussed in this paper must be borne in mind whenever salting-out is made use of in the fractionation of protein solutions and the purification of isolated fractions by re-solution and reprecipitation. Conclusions as to the homogeneity of proteins isolated by salt precipitation must also be reconsidered in the light of these results.

REFERENCES.

de Bruyn (1900), *Zeitsch. physikal Chem.* **32**, 63.
Chick (1913), *Biochem. J.* **7**, 318.
—— and Martin (1912), *J. Physiol.* **45**, 261.
Hardy (1900), *Proc. Roy. Soc.* **66**, 110.
Hofmeister (1888), *Arch. exp. Path. Pharm.* **24**, 247.
—— (1889), *Arch. exp. Path. Pharm.* **25**, 1.
—— (1891), *Arch. exp. Path. Pharm.* **28**, 210.
Hopkins (1899–1900), *J. Physiol.* **25**, 306.
—— and Pinkus (1898), *J. Physiol.* **23**, 130.
Kauder (1886), *Arch. exp. Path. Pharm.* **20**, 411.
Lewith (1888), *Arch. exp. Path. Pharm.* **24**, 1.
Mellanby (1907), *J. Physiol.* **36**, 288.
Michaelis and Mostynski (1910), *Biochem. Zeitsch.* **24**, 79.
—— and Rona (1909), *Biochem. Zeitsch.* **18**, 318.
—— —— (1910), *Biochem. Zeitsch.* **27**, 38.
Pauli (1898), *Pflüger's Archiv,* **71**, 333.
—— and Rona (1902), *Beiträge,* **2**, 1.
Posternak (1901, 1), *Ann. Inst. Past.* **15**, 85.
—— (1901, 2), *Ann. Inst. Past.* **15**, 169.
Spiro (1904), *Beiträge,* **4**, 300.

XL. SOME OBSERVATIONS ON THE ESTIMATION OF UREA.

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The chief aim of the following investigation was to find out a simpler method for the estimation of urea than those at present in use. The hypobromite method, although rapid and simple, has been shown to be unreliable [Mörner, 1903]. All the more accurate methods depend like that of Folin [1901, 1902] on the conversion of urea into an ammonium salt, and the estimation of the ammonia by distillation. These methods necessarily involve a preliminary estimation of the preformed ammonia of the urine. No useful purpose would be served by an enumeration of the chief modifications which the methods have undergone. Reference may be made to the exhaustive account given in the revised edition of Neubauer and Huppert's *Analyse des Harns* [1910].

The distillation of the preformed ammonia and that derived from the hydrolysis of urea occupies in each case about an hour. A considerable reduction of time and simplification of the method would be secured, if the distillation were replaced in both instances by titration in the presence of formaldehyde. The main objections to the latter method are that other amine derivatives, notably amino- and diamino-acids affect the result of the titration [Schiff, 1899, 1901, 1902], and that the best method for the determination of the neutral point, from which one starts, is still unsettled. Ronchese [1907] and Malfatti [1908] employ phenolphthalein throughout as indicator in their method for the estimation of ammonia in the urine by formol-titration, while Sörensen [1907, 1910] and Henriques [1909] use azolitmin paper as the indicator in neutralising the solution and phenolphthalein for the titration of the acids set free by the addition of neutral formol in their method for estimating amino-acids. De Jager [1909, 1; 1910, 1] follows the same procedure as Malfatti after adding excess of neutral potassium oxalate or of potassium sulphate. Folin [1903] had

previously made similar use of potassium oxalate in determining the acidity of the urine. The fact that solutions of chemically pure ammonium salts react acid to phenolphthalein has long been recognised. In consequence, the results of formol-titrations with this as the sole indicator are too low. One objection to the method of overcoming this difficulty adopted by Sörensen and Henriques is the inconvenience of the frequent testing with azolitmin paper required in the course of the titration. The addition of litmus to the solution renders the titration difficult and uncertain owing to the conflicting colour changes of the two indicators. In its place, I have used methyl-red [Rupp and Loose, 1908; Tizard, 1910; Howard and Pope, 1911] as the indicator of the neutral point and phenolphthalein for the determination of the amount of acid set free by the addition of neutral formol. Methyl-red has the advantage of being an exceedingly sensitive indicator for the titration of weak bases such as ammonia, while it is comparatively insensitive to weak acids. On the other hand, phenolphthalein is a good indicator for weak acids, but is not a reliable indicator for weak bases. The colour changes of the two indicators do not interfere with one another, and they may therefore be used together without any difficulty arising. Methyl-orange may be used instead of methyl-red, but it does not give such a sharp endpoint.

1. Preliminary Observations on the Titration of Pure Ammonium Salts.

The following experiments were carried out in order to ascertain the degree of accuracy of the method as compared with formol-titrations of ammonium salts in the presence of phenolphthalein alone. Five drops of a 0.5% alcoholic solution of phenolphthalein were added to 10 cc. of a decinormal solution of pure ammonium chloride, which had been previously rendered faintly acid by the addition of dilute hydrochloric acid. The solution was then neutralised by the addition of sufficient decinormal caustic potash just to render the solution faintly red. Excess (about 5 cc.) of neutral formol (50 cc. 40% formaldehyde, 1 cc. of 0.5% alcoholic phenolphthalein, and sufficient decinormal caustic potash to render the solution faintly pink) was then added, and the addition of decinormal alkali continued until the solution just acquired a faint permanent red colour. The addition of 9·55 cc. of decinormal alkali was found to be necessary to reach this point instead of the true value 10 cc. Two to three drops of a 0.2% alcoholic solution of methyl-red were added to another 10 cc. of a decinormal solution of

ammonium chloride, and then sufficient decinormal caustic potash just to convert the red colour of the solution to a yellow. On the addition of neutral formol, the solution again acquired a red colour owing to the hydrochloric acid, set free by the interaction of the formaldehyde with the ammonium chloride, acting upon the methyl-red. On the further gradual addition of a measured quantity of decinormal caustic potash, the red colour fades and is replaced by a yellow. The addition of alkali was continued until a faint permanent red colour, due to the action of the alkali on the phenolphthalein, just appeared. 9·95–10 cc. were required to reach this point. Repeated control estimations of the same character with solutions of pure ammonium sulphate as well as of ammonium chloride gave similar results. It will be noted that, when the two indicators are used, the solution during titration passes through three changes of colour. It is first red owing to the action of free mineral acid on the methyl-red, then orange to yellow when the solution contains only free organic acid, and finally red when just alkaline owing to the action of slight excess of free alkali on the phenolphthalein. The organic acid is formic acid present in the formaldehyde solution and resulting from the oxidation of part of the formaldehyde. Thus, prior to the addition of the neutral formol, the solution contains only ammonium chloride; while after its addition the fluid contains hexamethylenetetramine, free hydrochloric and formic acids, the latter two being in varying proportions according to the amount of formic acid present in the original solution of formaldehyde. In the presence of traces of free formic acid, methyl-red has nearly the same tint as in neutral solution, and, therefore, when all the free mineral acid has been neutralised, the colour of the methyl-red changes to faint orange and later to yellow, although the solution is found still to be distinctly acid to phenolphthalein.

2. Application of the foregoing results to the estimation of pure urea in aqueous solution.

Folin's method of hydrolysis [1901, 1902] is not applicable in this case, since the presence of a large amount of magnesium chloride interferes with the subsequent titration. A slight modification of Benedict and Gephart's method of hydrolysis [1909] was consequently adopted. The modification consisted merely in using more dilute acid for the hydrolysis of the urea. When a considerable excess of acid is used, the large amount of neutral salt formed in the course of neutralisation interferes with the accuracy of the determination of the endpoint [Michaelis and Rona, 1909; Andersen, 1911].

The use of a smaller amount of acid also lessens the risk of the decomposition of other nitrogenous substances, when the method is applied to urine. After using this method of hydrolysis for some time, I found that it had been previously employed by Henriques and Gammeltoft [1911].

At first the hydrolysis was carried out in sealed glass tubes heated in a glycerol bath to 160° for four hours. Two cc. of a $2·006\%$ solution of pure urea and 2 cc. of normal sulphuric acid were placed in a glass tube, which was then sealed and heated in a glycerol bath. After being allowed to cool, the sealed tube was opened and the contents carefully transferred to a flask, the broken tube being thoroughly washed with distilled water. The fluid was then neutralised in the presence of methyl-red as indicator, and the ammonia determined by means of formol-titration using phenol-phthalein as the indicator. The following are the results given in tabular form. One cc. of decinormal alkali is equivalent to 0·003 g. of urea.

Cc. of $2·006\%$ urea	Cc. of N. acid for hydrolysis	Cc. of N/10 alkali (formol titr.)	G. of urea found	Theoretical amount of urea
2	2 H$_2$SO$_4$	13·3–13·35	0·0399–0·04	0·0401
2	2·5 HCl	13·4	0·0402	0·0401

Since the use of sealed tubes is somewhat inconvenient, Benedict and Gephart's method [1909] of heating the solution in test-tubes covered with leadfoil and placed in an autoclave kept at a temperature of 155° for 1·5 hours was afterwards employed. A large number of estimations can readily be carried out simultaneously by means of this method. The temperature in the autoclave was varied in the first experiments in order to find the most suitable temperature for hydrolysis.

2·3101 g. of pure urea were placed in a 200 cc. flask, dissolved in water, 100 cc. of normal sulphuric acid were added, and the whole made up to 200 cc.; 5 cc. portions of the acid solution were then hydrolysed. The following table gives the results.

Temperature	Cc. of N/10 alkali for formol-titr.	G. of urea found	Theoretical g. of urea
144–145° (70 minutes)	18·8	0·0564	0·0577
152–153° (30 minutes longer)	(1) 19·15	0·05745	,,
	(2) 19·2	0·0576	,,
	(3) 19·15	0·05745	,,

Since the application of the foregoing results to the estimation of the urea in the urine involves a preliminary formol-titration of preformed ammonia and other amine derivatives, it was essential to test the method by applying it to the titration of amino-acids as well as to mixtures of amino-acids with ammonium salts.

3. Titration of amino-acids alone and with ammonium salts.

Glycine being easily prepared in a pure state, was selected as a type for the tests. Since one has to deal with a mineral acid solution in the final formol titration, it was obviously essential to carry out the estimation under similar conditions, and also to compare the results with those obtained when phenolphthalein was used as the sole indicator. The following are the results given in tabular form. The glycine solution was 0.71%.

These results indicate that, when the two indicators are used, the estimations of amino-acids and ammonium salts are more accurate than when phenolphthalein is the sole indicator. The fact that formol-titration yields too low results in the case of mixtures of amino-acids and ammonium salts was first noted by de Jager [1909, 2]. Henriques and Sörensen [1910] have suggested an explanation of this anomaly. More recently de Jager [1910, 2] has shown that the addition of urea enables one to obtain correct results. The latter fact is illustrated by the results given in (4) of the table.

Solutions used	Cc. N/10 alkali (for neutralisation)	Formol-titr. (found)	Theoretical result
(1) 10 cc. glycine 2·55 cc. N/10 HCl	2·55 (indicator methyl-red)	9·4	9·46
(2) 10 cc. glycine 2·75 cc. N/10 HCl	2·85–2·9 (indicator phenol-phthalein)	9·25	,,
(3) 10 cc. glycine 10 cc. N/10 NH$_4$Cl 2 cc. N/10 HCl	2 cc. (methyl-red)	18·65	19·46
(4) 10 cc. glycine 10 cc. N/10 NH$_4$Cl 2 cc. N/10 HCl 0·5 g. urea	2 cc. (methyl-red)	19·4	,,

4. Estimation of urea in urine.

(1) *Without a preliminary purification of the urea.*

Since the presence of phosphates renders the neutral point indefinite for both indicators, and since the neutral points for the two indicators are markedly different in solutions of phosphates, it was necessary to precipitate the phosphates with baryta mixture. The addition of an unduly large excess of barium chloride is to be avoided, as its presence interferes slightly with the accuracy of the titration; but a small excess is immaterial. When sulphuric acid is used for the hydrolysis of the urea the excess of barium present in the filtrate is precipitated as barium sulphate, and it

was mainly for this reason that normal sulphuric acid rather than hydro-chloric acid was used for the majority of the hydrolyses. The results of the method of formol-titration were controlled by comparison with the values for ammonia obtained by distillation with magnesia and with barium hydrate.

100 cc. of normal urine were mixed with 10 cc. of a binormal solution of barium chloride and sufficient barium hydrate to make the mixture distinctly alkaline, and finally made up with water to 200 cc. After the fluid had been thoroughly shaken, it was allowed to stand either for a few minutes or preferably in the presence of toluene for twelve hours. Henriques' method [1909] of freeing the urine from phosphates, sulphates and carbonates may be used as an alternative to the foregoing. The solution was then filtered. 25 cc. of the filtrate required 18·5 cc. of decinormal hydrochloric acid to render it neutral to methyl-red. The endpoint is not quite sharp. Excess of neutral formol (about 5 cc.) was added, and the titration with decinormal alkali completed. The average of two titrations was 5·2 cc. of decinormal alkali. This result gives the approximate amount of nitrogen in the form of preformed ammonia and amino-acids in 12·5 cc. of urine.

A number of 10 cc. portions of the filtrate were then mixed with 7–8 cc. of normal sulphuric acid, and placed in test-tubes in the autoclave, which was kept at a temperature of 155° for 1·5 hours. Although the fluid had darkened considerably in colour as a result of the heating with acid, the colour on dilution was not so deep as to interfere appreciably with the subsequent formol-titration. The ammonia in some portions was then set free by the addition of magnesia or barium hydrate, distilled off, and absorbed by excess of decinormal sulphuric acid (50–100 cc.) placed in the receiver. In others the ammonia and amino-acids were determined by formol-titration. Prior to carrying out the formol-titration it is advisable although not essential to filter off the barium sulphate. A considerable amount of pigment adheres to this and is removed with it. The test-tube and precipitate were then thoroughly washed, and the collected filtrate and washings treated by formol-titration. The following table (I) gives the results obtained. The deduction referred to in the table corresponds with the amount of substances in the original urine capable of titration by the formol method, consisting mainly of preformed ammonia and amino-acids.

An examination of the table indicates that the results of the formol-titration are invariably higher than those obtained by distillation; but agree well with one another. The difference is to be explained by the presence of non-volatile amino-groups set free by the hydrolysis with acid,

which raise the results obtained by formol-titration; but obviously cannot affect the values for ammonia obtained by distillation. The hydrolysis with normal acid splits up hippuric acid and any polypeptides which may be present, and thus renders the results of formol-titration higher than those obtained by distillation. The preliminary formol-titration, on which the deduction is based, gives the amount of nitrogen in the form of preformed ammonia and amino-acids: while, after hydrolysis, the final formol-titration gives the preformed ammonia and amino-acids, together with the amino-acids set free by hydrolysis, and the ammonia resulting from the decomposition of the urea. The difference between these two values represents the ammonia derived from urea plus the nitrogen of any amino-acids derived from hydrolysis of polypeptides and of hippuric acid. On the other hand, the method of distillation gives only the preformed ammonia and that formed by hydrolysis of urea. In calculating the results given in the table, the same deduction was made from the values obtained by distillation as from those by formol-titration. Strictly speaking, a smaller deduction should have been made from the latter. An independent determination of the preformed ammonia would be required to ascertain the true value of this correction. This point will be dealt with in a later part of this paper.

TABLE I.

(The quantity of urine was 5 cc. for each estimation.)

Method	Cc. of N/10 alkali	Deduction	% of urea	% of urea-N.
Formol-titration	(1) 32·15	2·1 cc. N/10 alkali	1·803	0·8414
,,	(2) 32·1	2·1	1·800	0·840
,,	(3) 32·4	2·1	1·818	0·848
Distillation with barium hydrate	(1) 32·05	2·1	1·797	0·8386
,,	(2) 32	2·1	1·794	0·837
,,	(3) 31·9	2·1	1·788	0·834
Distillation with MgO	(1) 31·7	2·1	1·776	0·829
,, ,,	(2) 31·75	2·1	1·779	0·830

The difference between the result obtained by distillation and that by formol-titration gives an approximate value for the non-volatile amino-derivatives. A more accurate estimate might be made by taking advantage of the fact ascertained by de Jager [1910, 2] that the addition of urea is necessary in order to secure the true result for the sum of amino-acids and ammonia. This addition is unnecessary in the first formol-titration, since sufficient urea is present in the urine; but after hydrolysis the addition

of pure urea to the neutralised solution would be necessary before carrying
out the second formol-titration. I have not considered the correction of
sufficient importance in the case of the normal urines examined to warrant
its introduction into the table; but in the event of a marked difference
being found between the values obtained by distillation and formol-titration
indicating excess of amino-acids, it would be advisable to make the
correction.

The next table (II) gives the results of a similar series of estimations in
a sample of another urine. Normal hydrochloric acid was used in this
instance instead of sulphuric.

The same features will be noted as in the previous table. When barium
hydrate is used as the fixed alkali, the values for ammonia are usually
higher than when magnesia is employed. In order to obtain constant
results, the distillation has to be more prolonged in the case of magnesia
than with barium hydrate. The distillation usually occupied 1–2 hours
in both cases. The differences between the results of formol-titration and
of distillation is greater than in the other urine.

TABLE II.

(The quantity of urine used for each estimation was 5 cc.)

Method	Cc. of N/10 alkali	Deduction	% of urea	% of urea-N.
Formol-titration	(1) 36·65	2·5	2·049	0·9562
,,	(2) 36·6	,,	2·046	0·9548
,,	(3) 36·6	,,	2·046	0·9548
Distillation with barium hydrate	(1) 35·45	2·5	1·977	0·9226
,,	(2) 35·65	,,	1·989	0·9282
,,	(3) 35·75	,,	1·995	0·931
Distillation with MgO	(1) 35	2·5	1·95	0·910
,, ,,	(2) 35·5	,,	1·98	0·924

(2) *Estimation of urea in urine after a preliminary extraction*
of the urea with a mixture of alcohol and ether.

Folin's [1901] and Benedict and Gephart's methods [1909], as well as all
other methods depending on the conversion of urea into the ammonium salt
of the acid used for hydrolysis, give inaccurate results when carbohydrates
are present in the urine. Mörner [1903] has shown that this is due to the
fact that the humin-substances formed by the action of the acid on the
sugar contain firmly bound nitrogen, when ammonium salts are present or
formed during the hydrolysis.

Mörner adopted the following method in order to obtain a solution of urea free from carbohydrates. The phosphates were precipitated from 5 cc. of urine by the addition of 1·5–2 g. finely powdered barium hydrate, and the fluid was then extracted with 100 cc. of a mixture of two volumes of alcohol and one of ether. After being thoroughly mixed, the solution was allowed to stand for 24 hours, then filtered, and the precipitate washed with the mixture of alcohol and ether, the filtrate and washings being collected in the flask afterwards to be employed in the distillation. The alcohol and ether were then distilled off, and the residue after being freed from ammonia by concentration at 50° after the addition of magnesia, was hydrolysed by Folin's method [1901]. I carried out the first stages of the method in the same way; but instead of freeing the aqueous solution from ammonia by the addition of magnesia, I determined the amount of amino-nitrogen by means of formol-titration. The method of hydrolysis adopted was the modification of Benedict and Gephart's method [1909] previously described. In some cases normal hydrochloric acid was used for the hydrolysis, in others normal sulphuric acid. The following table summarises the results.

TABLE III.

Method	Results in cc. of N/10 NH$_3$	Deduction	% urea	% urea-N.
1. Direct formol-titration	(a) 29·55	0·85	1·722	0·8036
	(b) 29·7	,,	1·731	0·8078
	(c) 29·65	,,	1·728	0·8064
2. Distillation with Ba(OH)$_2$	(a) 29·1	0·85	1·695	0·791
	(b) 29·15	,,	1·698	0·7924
3. Distillation with MgO	(a) 28·45	0·85	1·656	0·773
	(b) 28·5	,,	1·659	0·774
	(c) 28·1	,,	1·635	0·763

The following are the chief features revealed by an examination of the table. The comparatively low value of the deduction is to be explained by the fact that the method of preparing the aqueous solution of urea involves the loss of the greater part of the preformed ammonia. The values for urea obtained by the formol method are, as in the previous estimations, higher than those obtained by distillation. Amino-derivatives evidently pass into the alcohol and ether mixture. Hippuric acid is probably the most important of these. The glycine derived from its hydrolysis must raise the results of the formol-titration, while it obviously cannot affect the values obtained by distillation. The results obtained by distillation with magnesia are considerably lower than those with barium hydrate.

5. FINAL CONTROL OF THE METHOD BY AN INDEPENDENT DETERMINATION
OF THE PREFORMED AMMONIA.

The estimations hitherto given suffer from the defect of not having been controlled by an independent determination of the preformed ammonia. Reference was previously made to this fact. In a final series of experiments, I have estimated the total nitrogen by Kjeldahl's method, the nitrogen of the preformed ammonia by the method of Krüger, Reich, and Schittenhelm [1903], the ammonia and urea nitrogen after hydrolysis by distillation, and the nitrogen of the amino-acids by Henriques' method [1909]. The results for 100 cc. of urine are stated in Table IV in terms of decinormal alkali and in Table V as percentages.

TABLE IV.

Results for 100 *cc. urine expressed in terms of decinormal nitrogen.*

Total N.	Urea and NH$_3$-N. (distillation)	Urea + NH$_3$ + amino-acid nitrogen (formol-titration)	NH$_3$ + amino-N. (in original urine)	Ammonia-N. (distillation)
587	529	550	33·6 (Sörensen)	23·9
585	528	544	35·2 (methyl-red)	24·2
583	524	546	—	—
585 (mean)	527 (mean)	547 (mean)	—	24·05 (mean)

TABLE V.

Total N.	Urea-N.	Urea-N. (formol-titr.)	Amino-N.	Ammonia-N.
585	503	513 (Sörensen)	9·6 (Sörensen)	24·05
—	—	511·4 (methyl-red)	11·2 (methyl-red)	—
0·819 %	0·704 %	0·718 % (S.) 0·716 % (M.)	0·013 % (S.) 0·0157 % (M.)	0·0337 %

The value given in column 2 of Table V is arrived at by deducting the amount of preformed ammonia as determined by the method of Krüger, Reich, and Schittenhelm from the total amount of ammonia obtained by distillation after complete hydrolysis of the urea. This may be regarded as the correct value for urea arrived at by a standard method. The values for the nitrogen of urea given in the third column of Table V are obtained by deducting the results of column 4, Table IV, from those of column 3, Table IV, using in the first instance the results of Henriques and Sörensen's method and in the second case those obtained by the use of methyl-red and phenol-phthalein as the two indicators. The values for the nitrogen of the amino-acids given in column 4 of Table V are arrived at by subtracting the

results of the method of Krüger, Reich and Schittenhelm given in column 5 of Table IV from those of column 4, Table IV, using in the one case Henriques' method, and in the second formol-titration with methyl-red as the first indicator.

Sörensen [1907, 1910] used fifth-normal alkali for the titration of the amino-acids. Notwithstanding the fact that this strength is to be preferred for reasons stated by that author, I was obliged to continue the use of decinormal alkali as it had been used throughout the foregoing investigation. These final results indicate that the error in the method of estimating urea in this urine by formol-titration is a positive one amounting to approximately $1\cdot7\,^0/_0$.

The chief advantages of the formol-method of estimating urea are that it is not only more rapidly carried out than the methods involving distillation, but also entails less arrangement of apparatus. The fact that the method gives at the same time an approximate estimate of the preformed ammonia and amino-acids is also an advantage, which compensates to some extent for the less degree of accuracy attainable. These facts suggest that it might find useful application for the examination of urines in clinical laboratories as a substitute for the hypobromite method. Another possible application of the method might be to the study of the hydrolysis of urea by enzymes and micro-organisms.

REFERENCES.

Andersen (1911), *Skand. Arch. Physiol.* 25, 104.

Benedict and Gephart (1909), *J. Amer. Chem. Soc.* 30, 1760.

De Jager (1909, 1), *Zeitsch. physiol. Chem.* 62, 333.

—— (1909, 2), *Zeitsch. physiol. Chem.* 64, 337.

—— (1910, 1), *Zeitsch. physiol. Chem.* 67, 1.

—— (1910, 2), *Zeitsch. physiol. Chem.* 67, 107.

Folin (1901), *Zeitsch. physiol. Chem.* 32, 504.

—— (1902), *Zeitsch. physiol. Chem.* 36, 333.

—— (1903), *Amer. J. Physiol.* 9, 263.

Henriques (1909), *Zeitsch. physiol. Chem.* 60, 1.

—— and Gammeltoft (1911), *Skand. Arch. physiol.* 25, 153.

—— and Sörensen (1910), *Zeitsch. physiol. Chem.* 64, 120.

Howard and Pope (1911), *J. Chem. Soc.* 99, 1333.

Krüger, Reich, and Schittenhelm (1903), *Zeitsch. physiol. Chem.* 39, 73, 165.

Malfatti (1908), *Zeitsch. anal. Chem.* 47, 273.

Michaelis and Rona (1909), *Biochem. Zeitsch.* 23, 61.

Mörner (1903), *Skand. Arch. Physiol.* 14, 321.

Neubauer and Huppert (1910), Edition 11, pp. 560–577.

Ronchese (1907), *J. Pharm. Chim.* (6), 25, 611.

Rupp and Loose (1908), *Ber.* 41, 3905.

Schiff (1899), *Annalen,* 310, 25.

—— (1901), *Annalen,* 319, 59, 287.

—— (1902), *Annalen,* 325, 348.

Sörensen (1907), *Biochem. Zeitsch.* 7, 45.

—— (1910), *Biochem. Zeitsch.* 25, 1.

Tizard (1910), *J. Chem. Soc.* 97, 2485.

XLI. GAS-ELECTRODE FOR GENERAL USE.

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The standard forms of hydrogen-electrode, such as those used by Dolezalek [1899], Wilsmore [1900] and Bjerrum [1910] and, for fluids containing carbon dioxide, Hasselbalch [1910], must remain indispensable when physical measurements of the highest order of accuracy are undertaken.

With these, after the expenditure of considerable time and care, and with continued vigilance over every part of the electrical apparatus, consecutive results concordant to about 0·05 millivolt can sometimes be obtained. But, for these results to have any meaning beyond the nearest millivolt or two, it is essential that the diffusion potential difference of the cell should be accurately known in every determination: and as this is recognised, by Cumming and others whose investigations continue to throw light on this difficult subject, to be almost impossible, it follows that, as far as absolute H˙ ion determinations are concerned, attention should be focussed more particularly on diffusion potential errors (in view of their greater magnitude) than on those found at the electrode itself.

In general laboratory practice, therefore, especially when dealing with protein-containing materials, which may or may not be free from carbon dioxide, the most suitable electrode is not one which, when coupled with an ideal electrical apparatus, will give results of this high order of accuracy, regardless of the expenditure of time and material. Rather it is one which, without exaggerating appreciably the diffusion potential error, will give, under ordinary working conditions and in a few minutes, results correct to 1 millivolt or so on one or two cc. of fluid without loss or contamination.

Anticipating a long series of H˙ ion determinations on protein solutions, some of them containing carbon dioxide, I endeavoured to devise such an electrode. The arrangement subsequently adopted and the experiments made to discover its imperfections are described below. Only one form of

apparatus has been used for all cases. When dealing with a solution containing carbon dioxide or other dissolved gases a slight modification of technique is all that is necessary.

APPARATUS.

The modifications of existing apparatus which I have employed consist of
- (1)   the electrode vessel,
- (2)   the filling apparatus,
- (3)   the support in the constant temperature bath.

The electrode vessel (ABD, Fig. 1) is somewhat more complicated than the simple **V** shape generally employed, but this is amply compensated for by the simplicity of the other parts of the apparatus and the many conveniences accompanying its use. I have used throughout the platinum point advocated by Michaelis, just making contact with the surface of the fluid. The platinum point is mounted at the end of a glass tube A. The protruding end is blackened in the usual manner. The other end makes contact with a small globule of mercury placed inside the tube.

The lower end of A may be ground to fit the outer tube B or else a joint may be made by means of a rubber stopper as in the diagram. Attached to B at the side is a very fine bore capillary tube carrying a tap D, and at the lower end is a second capillary tube not quite so fine, of about 1 mm. bore. At the lower end of this may be fitted a very small glass stopper, but it is not necessary and has certain disadvantages.

The filling apparatus used with all these electrode vessels is depicted together with other apparatus in Fig. 2. It consists of a three-way piece F connecting a 5 cc all-glass syringe G, well lubricated with vaseline, a short piece of fine-bore stout rubber tubing H and a glass tube carrying a glass stopcock which is connected with the hydrogen supply.

As *support* for the electrode vessels in the constant temperature bath, while taking potentiometer readings, I have used a glass trough K (Fig. 1) in which the electrode vessels stand side by side in a suitable connecting solution. For single electrode vessels a large test tube serves excellently. The other half-electrode employed, a tube from which leads into the glass trough, is also immersed in the water-bath.

I have used principally the calomel-saturated potassium chloride half-electrode recently described by Michaelis and Davidhoff [1912] and a standard calomel N/10 KCl half-electrode. The connecting solutions were saturated potassium chloride solution, saturated ammonium nitrate [Cumming, 1907].

and solutions of potassium chloride 1·75 N and 3·5 N respectively, employed for the extrapolation method of Bjerrum.

In some experiments on diffusion potential two gas electrodes of the vertical type were stood side by side in the trough containing connecting solution.

EXPERIMENTS WITH SOLUTIONS FREE FROM CARBON DIOXIDE AND OTHER DISSOLVED GASES.

Technique. The electrode vessel is first of all connected by the capillary side tube *D* (Fig. 1) to the rubber tube of the filling apparatus (shown at *GFH*, Fig. 2), and hydrogen passed for a few seconds, both glass taps being open. Then, by alternately drawing out the piston with the tap on the electrode vessel shut, and pushing it home with the tap open, the last traces of air are expelled from the dead space of the syringe and the T-piece. The lower end of the electrode vessel is now brought under the surface of a sample of the fluid to be examined and the glass tap on the T-piece shut. By pulling out the plunger of the syringe the fluid is drawn up until its surface is just at the point where the platinum point is sealed to the glass. Slight movements of the plunger in and out now cause the liquid by rising and falling to wash the platinum point well without wetting the tube *A* (Fig. 1) supporting it. Finally the height of the liquid is adjusted till the point just touches the surface, the glass tap *D* on the side capillary closed, and the vessel containing the remainder of the sample of fluid taken away.

The electrode vessel is now disconnected from the filling apparatus, and the lower capillary wiped dry with a piece of clean filter-paper and placed in the glass trough in a constant temperature bath. There is sufficient connecting solution in the trough to cover the electrode vessel above the rubber stopper. The two copper wires from the potentiometer are now led, one into the calomel half-electrode, and the other into the tube *A* and the reading is taken from which the value of P_H^+ (the negative exponent of the H˙ ion concentration) may be calculated directly.

In order to be certain that the platinum electrode is working properly and that equilibrium is established instantaneously, a few minutes at least may be allowed to elapse before a second reading is taken. Meanwhile the filling apparatus can be used to fill another electrode vessel with another sample, and when this is introduced into the trough, the copper wire can be changed over from the first electrode vessel and a reading of that taken. While the equilibrium of the second electrode is being checked, the first can

be refilled. It is removed, dipped into a large beaker of distilled water, and dried externally with a clean towel or filter paper and connected again to the filling apparatus.
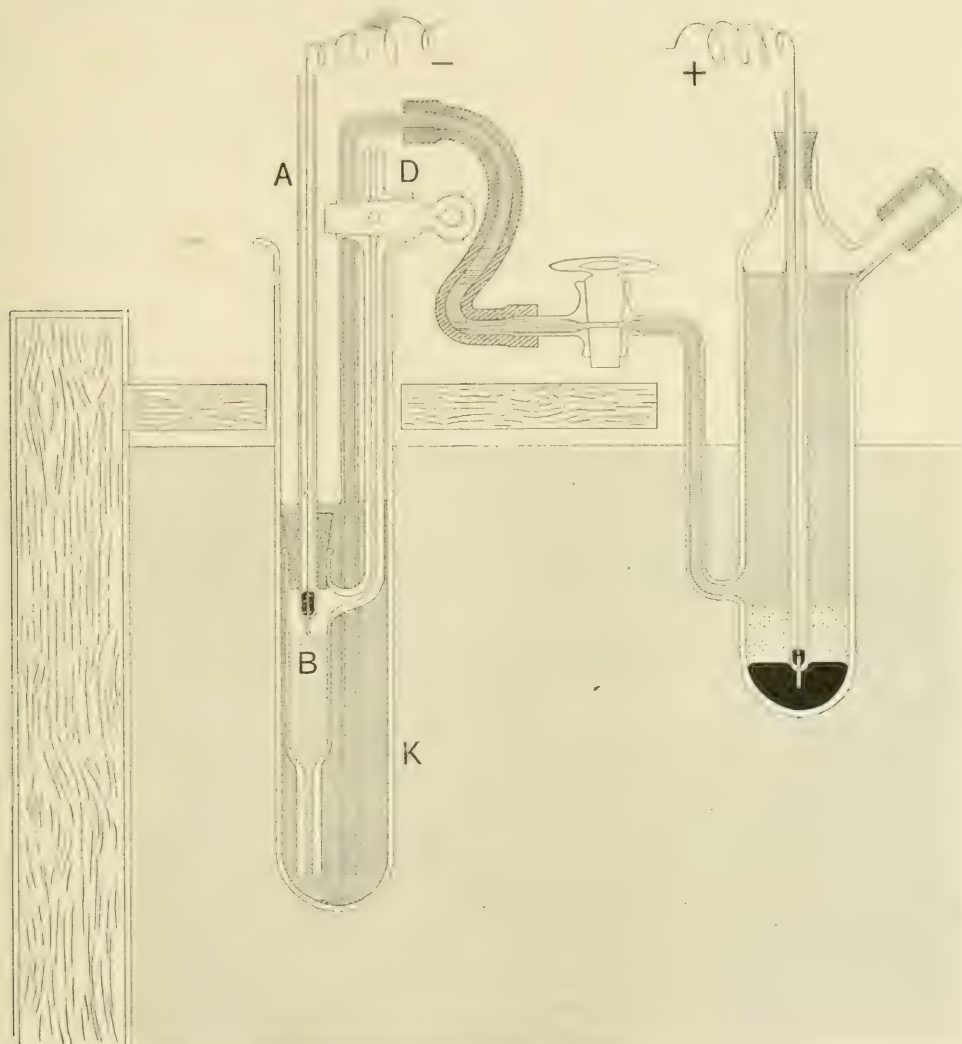
Fig. 1.

Opening the tap *D* (Fig. 1) and pushing home the syringe, the electrode vessel is emptied, and the tap on the filling apparatus opened for a few seconds to fill it with fresh hydrogen. The next sample having been brought underneath the electrode vessel, the tap on the three-way piece is closed.

liquid drawn up by the syringe, expelled so as to get rid of traces of the previous sample, and then the vessel filled with a fresh sample as before.

If an electrode is properly blacked, and in good working order, it should give the equilibrium value immediately and scarcely change afterwards—two millivolts at most—during the next ten or twenty minutes. Diffusion of hydrogen into and through the rubber stopper causes slight inconvenience if the electrode has not been used for a day or two. The liquid rises in the vessel and the platinum becomes immersed beyond the point. Equilibrium under these conditions would not be reached for a long time. To rectify this, the glass tube A can be slid upwards through the rubber stopper. It is advisable when setting the electrode aside, to push the glass tube holding the electrode well through the cork, fill with hydrogen, suck up distilled water till the platinum is immersed and after disconnection from the filling apparatus leave the vessel immersed in distilled water.

Washing out of electrode vessel. One rinsing only between two samples has been found sufficient. Several experiments were performed to demonstrate this. One set of figures is given below.

Temp. 18°. Bar. 750 mm. Half-electrode sat. KCl-calomel. Connecting fluid sat. KCl. Calculated P_H^+ value for 0·0993 N HCl (dissociation 91·6 %) = 1·041. Calculated P_H^+ value 0·1 N NaOH (14·14 − 1·075) = 13·065. Sample used was known not to be absolutely free from CO_2.

Solution used	Volume used	Remarks	Time of observation	Potential π	$P_H^+ = \dfrac{\pi - 0.251}{0.0577}$
0·0993 N HCl	2 cc.	Apparatus at commencement clean and dry	8.45	0·3110	1·039
			8.50	0·3110	1·039
			9.10	0·3113	1·045
,,	4 cc.	Used 2 cc. for rinsing out last sample	1.45	0·3113	1·045
			2.00	0·3115	1·048
			2.15	0·3120	1·057
			2.45	0·3120	1·057
0·1 N NaOH	4 cc.	Used 2 cc. for rinsing out last sample	2.50	1·0020	13·015
			2.55	1·0022	13·019
			3.15	1·0022	13·019
			3.50	1·0030	13·032
0·0993 HCl	4 cc.	Used 2 cc. for rinsing out last sample	4.19	0·3115	1·048
,,	circ. 20 cc.	Rinsed out many times and filled side capillary, see page 425. Passed bubble for 5 mins.	4.45	0·3105	1·031
			4.55	0·3110	1·039
			5.00	0·3110	1·039
			next morning		
			8.5	0·3108	1·036

Temperature. Although most of these determinations have been made at 18° exactly, it is unnecessary to pay too great attention to this point as long as the temperature of the calomel-saturated KCl half-electrode and the gas

electrode are the same in any one determination. Calculation from the constants given by Michaelis for this combination demonstrates that the error in measurement of the value of P_H^+ per $1°$ is 0.007 for a neutral solution the H˙ ion concentration of which has no temperature coefficient. The error with a N/10 KCl-calomel electrode is about three times this. Approximate determinations with the saturated KCl half-electrode may quite well be made on the open bench without a constant temperature bath.

When a N/10 KCl-calomel half-electrode or a N/10 HCl-hydrogen half-electrode are used in combination with the gas electrode, more careful temperature adjustment is necessary. As a direct result of the diminutive size of the electrode vessel, and the small quantity of fluid used therein, the time taken to arrive at temperature equilibrium is very short.

Quantity of material used. The size of electrode vessel found most convenient for general use has the following principal dimensions : length of tube *B* (Fig. 1) 75 mm., maximum external diameter 10.5 mm., length of lower capillary 25 mm. The capacity when full is 1.5 cc., so that a determination can be performed easily on 2 cc. of the material if the electrode vessel be clean and dry to start with. When the electrode vessel is not cleaned and dried between two determinations, 4 cc. are required—2 cc. to rinse out the vessel and 2 cc. from which the electrode vessel is filled.

If only smaller quantities of material are available, a smaller electrode vessel may be used. Quite good results were obtained by one holding 0.3 cc. This is not necessary, however, for the quantity of material available may be drawn up into a vessel of the most convenient size and then saturated potassium chloride drawn up afterwards until contact is made with the platinum point. Mixing of the two fluids only takes place very slowly by diffusion unless the density of the fluid examined approximates to that of saturated KCl. In those cases when it is known that the H˙ ion concentration does not change by such treatment the sample may be diluted with pure water [cf. Michaelis and Davidhoff, 1912]. This is the substitution of an indirect method for a direct one and so an additional possibility of error is introduced.

The extent of the contamination of the fluid during a determination is very small since the boundary surface between it and the saturated potassium chloride solution is only that of a section of the capillary tube. When a very small glass stopper is fitted at the bottom of the capillary, possibility of contamination is still further removed. It is usually found that enough potassium chloride diffuses round the stopper to diminish the resistance sufficiently for a reading, not always very sharp, to be taken.

In many cases, therefore, where a small quantity of liquid only is available, it is possible to determine its reaction electrometrically and recover it with very little contamination, dilution, or loss, for further experiments. Usually a moderate economy of material is advantageous and, on this and other grounds, the use of the vertical electrode filled by suction has been found by me much more convenient than the **V**-type for general work. Frothing fluids do not exhibit their disagreeable characteristic, as hydrogen is not bubbled through them, but they are forced up by external atmospheric pressure. There is no need, therefore, to make an indefinite contact with the froth. The abolition of tapes or wool threads soaked in potassium chloride solution was found a great convenience.

Diffusion Potential. In the electrolytic cells of which one half-electrode is hydrogen in contact with an aqueous solution there are really four differences of potential involved. There are the two electrode potentials proper and two differences of potential where the electrode solutions come into contact with the connecting solution. The latter differences of potential are for the most part dependent upon the natures of the electrode solutions and of the connecting solution, but the time the solutions have been in contact is a concomitant factor. Cumming and Gilchrist [1913] have recently investigated this quantitatively and find that this "time change" is more marked with capillaries than open tubes. They, therefore, recommend that capillaries should be avoided in the construction of an electrolytic cell; so also should membranes, cotton wool plugs and, presumably, tapes.

In the vertical electrode vessel filled by suction, the surface between the solution examined and the connecting fluid is in a capillary or at the end of one. It may readily be made to occur at the wider part of the tube (p. 415, paragraph 4). I have made a number of careful measurements with this cell to determine how far variations in potential difference can be traced to boundary changes, and to what extent these alter when the surface between the electrode solution and the connecting solution is in the broader part of the tube instead of at the end of the capillary.

Using N HCl and N/10 HCl as electrode solutions connected to a saturated KCl-calomel half-electrode by saturated KCl solution, I could not detect with certainty any difference in the time change over half an hour in each case—first with boundary at end of capillary, second with boundary in broader part of the tube. Over longer periods, differences in the "time changes" could probably be detected, but do not rightly enter into consideration in the ordinary use of the electrode.

I have sometimes used saturated ammonium nitrate [Cumming, 1907]

instead of saturated potassium chloride as connecting fluid when examining acid solutions. With the N/10 KCl-calomel half-electrode N and N/10 HCl gave the potentials 0·3390 and 0·3974 respectively; calculated values 0·3407, 0·3977. With the saturated KCl-calomel electrode, however, the results were about 18 millivolts too low, the contact potential between saturated potassium chloride solution and saturated ammonium nitrate being of that order.

Comparison between the results obtained with the vertical and the
V-electrode vessels.

Both electrode vessels were fitted with rubber stoppers through which passed the glass tube in the end of which the blackened platinum point was mounted. When necessary this was adjusted so as just to touch the surface of the liquid. Temp. 18°. Connecting solution saturated KCl solution.

Date	Solution	Half-electrode			V-electrode vessel	Vertical electrode vessel	Previous results
						Potential readings	
18 April	0·01 Na₂CO₃ Aq.	Calomel.	Saturated KCl		0·8770	0·8780	—
11 April	Sodium Citrate (Sörensen)	,,	,,	,,	0·6240	0·6235	0·6238 (Sörensen)
6 May	Standard Acetate solution (Michaelis)	,,	,,	,,	0·5165	0·5170	0·5175 (Michaelis)
7 May	0·1 N HCl	,,	,,	,,	0·3122	0·3125	—
8 May	0·1 N HCl	,,	,,	,,	0·3122	0·3125	—
15 May	N HCl	,,	0·1 N KCl		0·3435	0·3425	—
15 May	N HCl	,,	Saturated KCl		0·2558	0·2560	—

ELECTROMETRIC TITRATION.

In acidimetry and its various applications the addition of a standard alkaline solution is continued until the titrated fluid has a certain H˙ concentration previously agreed upon. This is called the " end point." Generally the " titre " or amount of standard alkali which must be added to a certain amount of the fluid to be titrated in order to arrive at the " end point" is all the information required. The observer, with a proper understanding of the nature of the reaction, knows the correct " end point " for the object he has in view, and chooses an indicator which will tell him by its colour change when the " end point " is reached.

For instance, in the titration of an acetic acid solution it is desired to add standard NaOH solution until all the acetic acid is converted to sodium acetate. The hydrogen-ion concentration of sodium acetate solution is about $P_H^+ = 7·5$ to 9·5 so that phenolphthalein or another indicator exhibiting its colour change over this range of reaction is employed.

Again, in the Kjeldhal titration N/10 NaOH is added to a very dilute solution of sulphuric acid containing some ammonium sulphate. The "end point" is reached when all the sulphuric acid is converted to ammonium sulphate and sodium sulphate. Ammonium sulphate is faintly acid so that a trace of soda in excess will bring the mixture nearly neutral. Hence an indicator is chosen which is very sensitive at the neutral point—methyl orange, alizarin, rosolic acid have all been recommended. Such simple titration processes depend for their accuracy and reliability on a sudden change taking place in the reaction of the titrated fluid at some point during the addition of the alkaline solution, and the correct choice of an indicator which demonstrates this change. When dealing with very feebly dissociated acids and bases, of which proteins and some of their products of hydrolysis are excellent examples, such sudden changes of reaction do not occur. The titration process becomes more difficult. Moreover at any time it gives no information as to the rate of change of concentration of H˙ ions taking place progressively step by step as the alkaline solution is added. The measurement of this is important, and its neglect is frequently the outcome of the fact that it is a laborious process. To do it colorimetrically would be tedious and, in small quantities of fluid, impossible. Electrometrically with an electrode vessel of the standard type and a tape it would be impracticable, since loss and contamination of the fluid by potassium chloride would inevitably occur. With a vertical electrode vessel filled by suction these measurements of change of H˙ ion concentration during the titration process have been found quite practicable. There is no loss, practically no contamination by potassium chloride, and each determination of H˙ ion concentration during the titration process takes about two minutes.

Apparatus. Fig. 2 is a scale drawing of the apparatus used for titrating 10 cc. quantities. A small beaker contains the fluid to be titrated and dipping into it is a small electrode vessel (*B*) of capacity 3 cc. and a capillary tube filled with saturated KCl solution, connected to the saturated KCl-calomel electrode. Passage of liquid bodily along the capillary is prevented by the insertion of a long tightly packed plug of cotton wool, and if necessary a clip on the rubber tube connecting it to the half-electrode. The conductivity of the potassium chloride is so good that readings may still be taken with the clip on and sometimes, if the tap on the half-electrode be not vaselined, with this tap shut. A standard burette is clamped above the beaker. Two wires from the potentiometer lead to the half element and the electrode vessel respectively.

Technique. The electrode vessel is filled with hydrogen, and, while the

Hydrogen →
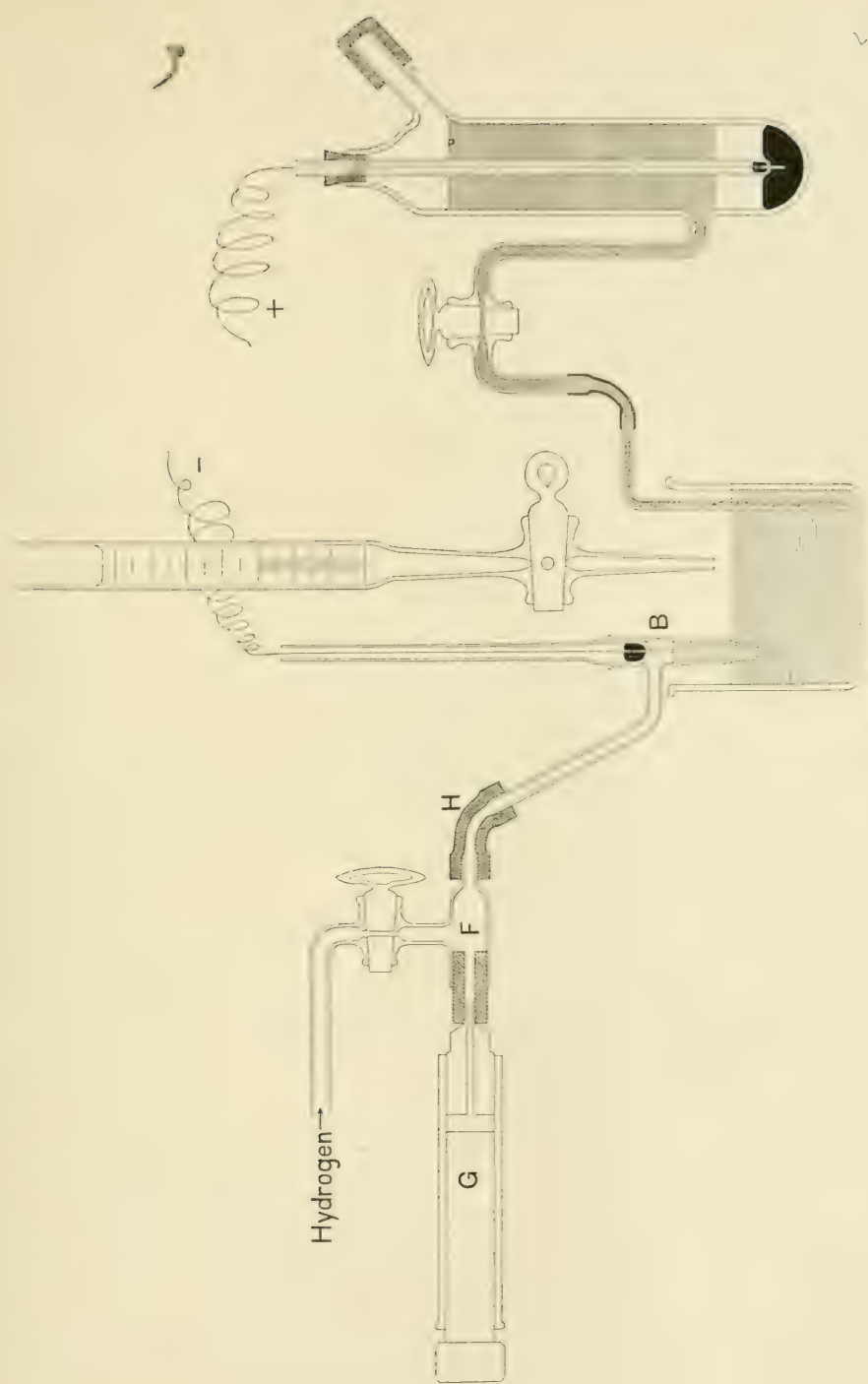
Fig. 2.

gas is still passing, its lower end brought below the surface of the measured quantity of liquid to be titrated which has been introduced into the beaker. The tap on the filling apparatus is turned off and by withdrawing the plunger, liquid is sucked up until contact is made. A reading of the potential indicates the reaction of the original liquid. The plunger is pushed home, a few drops of the titrating solution in the burette are run in, and by imparting a rotary motion to the beaker its contents are mixed until uniform throughout, and the reaction determined as before. In practice it is found best to draw the liquid up into the side tube and push it out several times in order to rinse the platinum point well between each reading.

From the results a curve may be plotted showing the relationship between the quantity of fluid added and the reaction. If any doubt exists as to the accuracy of the results obtained in this way, one or two points on the curve can be checked by single additions of N/10 alkali followed by determinations in a larger electrode vessel.

Example 1. 10 cc. of N/10 glycocoll solution containing in 1 litre 7·505 g. glycocoll and 5·85 g. sodium chloride [Sörensen, 1909, 1] were titrated with N/10 HCl electrometrically. Two separate titrations A and B were performed and subsequently the amount of potassium chloride accidentally introduced was found to be equivalent to 3·3 cc. of decinormal solution in one case and 3·0 cc. in the other, though no clip was used and the tap was open. The 10 determinations in titration B took 13 minutes; the 21 determinations in titration A took 43 minutes, though no particular attempt was made at speed.

Electrometric titrations of 10 cc. *glycocoll solution with* N/10 *HCl.*

Connecting fluid—saturated KCl solution.   Half-electrode—saturated KCl-calomel.   No correction for diffusion potential.

Vol. N/10 HCl added	E. M. F. in titration A	E. M. F. in titration B	Calculated from Sörensen's results
1 cc.	0·446, 0·445, 0·445	0·443	0·446
2	0·426, 0·426, 0·426	0·425	0·426
3	0·413, 0·414, 0·414	0·412	0·413
4	0·404, 0·404	0·402	0·403
5	0·394, 0·394	0·393	0·394
6	0·387, 0·387	0·386	0·386
7	0·381	0·379	0·379
8	0·374	0·373	0·373
9	0·369	0·367	0·367
10	0·365, 0·365, 0·365	0·362	0·362

Example 2. Advantage was taken of the opportunity now afforded of following the changes of reaction during a Sörensen formaldehyde titration.

A 4 % solution of Witte peptone was employed and the titration performed with every care. First colorimetrically, compensating for the colour of the peptone solution by a special tintometer, and then electrometrically.

1. SÖRENSEN TITRATION OF 4 % WITTE PEPTONE SOLUTION COLORIMETRICALLY.

Stage (1). *The neutralisation of the peptone solution.* Neutral red solution was used as indicator, and as a standard of neutrality a mixture of N/15 phosphate solutions (34·4 cc. acid + 65·6 cc. alkaline). 10 cc. of this solution —its reaction does not change on dilution [Szili, 1904]—to which 1 cc. of neutral red solution was added, were placed in a cylindrical vessel with a flat bottom D. A similar vessel surmounting this contained 10 cc. of Witte peptone B. Two corresponding vessels, A and C, contained distilled water, and 10 cc. Witte peptone solution plus 1 cc. neutral red solution respectively. Looking down AC the tint appeared redder than that seen looking down BD, and successive additions of N/10 NaOH solution were made until 0·5 cc. had been added, and the two columns were seen to match. It was considered that the 10 cc. of Witte peptone solution were neutralised by the addition of 0·5 cc. N/10 NaOH. Unfortunately the colorimetric result is misleading here. The P_H^+ value is 7·66 and not 7·07. The errors in P_H^+ found by Sörensen [1909, 2] in similar determinations with 2 per cent. Witte peptone solution containing 0·1 N NaCl and correcting for the colour of the solution by 2 drops of Bismarck brown and 2 drops of helianthin II were 0·18 and 0·12 in the same direction in two cases. Rosolic acid gave a similar result.

Stage (2). *Titration in presence of formaldehyde.* The problem is to discover how much 0·1 N NaOH must be added to a mixture of 10 cc. of Witte peptone solution and 10 cc. of neutral 40 per cent. formaldehyde solution in order that the resulting mixture should have the reaction $P_H^+ = 8·68$ (7 borate + 3 HCl); what further addition must be made to bring P_H^+ value to 8·91 (8 borate + 2 HCl); and what still further addition will bring the value to $P_H^+ = 9·09$ (9 borate + 1 HCl).

Using the same apparatus the solutions were arranged as shown on p. 422.

Formaldehyde bleaches slightly the colour of Witte peptone so that its colour must be compensated for by a solution similarly bleached. 1 cc. saturated sodium chloride solution is added to each formaldehyde-peptone mixture to inhibit the formation of polymethylimino-compounds. The same quantity was added to the 20 cc. of Sörensen mixture in D in order that the "neutral salt effect" on the phenolphthalein should be of the same order in

A.

10 cc. Witte peptone solution.
10 cc. formaldehyde containing 1·0 cc. of
   0·1 % phenolphthalein in 50 % alcohol.
1 cc. saturated NaCl solution.
(4·8 cc. 0·1 N NaOH were added to this tube
   to make a match.)

B.

10 cc. Witte peptone solution.
10 cc. formaldehyde solution.
 1 cc. saturated NaCl solution.

C.

20 cc. distilled water.

D.

20 cc. Sörensen mixture.
   (7 borate + 3 HCl.)
1·0 cc. of 0·1 % phenolphthalein in 50 %
   alcohol.
1 cc. saturated NaCl solution.

A and *D*. The amount of 0·1 N NaOH added to *A* before a match resulted was 4·8 cc. *D* was now removed and replaced by *D'* containing 20 cc. (8 borate + 2 HCl) mixture, 1·0 cc. of 0·1 % phenolphthalein in 50 % alcohol, and 1 cc. saturated sodium chloride solution. A further 0·3 cc. of 0·1 N NaOH was required in *A* to make a match. *D'* was now replaced by *D''* containing 20 cc. (9 borate + 1 HCl) mixture, 1·0 cc. of 0·1 % phenolphthalein in 50 % alcohol, 1 cc. saturated NaCl. A further 0·3 cc. of 0·1 N NaOH was required to make a match. Hence by a colorimetric method it has been found that the following mixtures have the corresponding H⁺ ion concentrations given in the table:

P_H^+
7·07   10 cc. Witte peptone solution + 0·5 cc. 0·1 N NaOH
8·68   ,,   ,,   ,,   + 10 cc. neutral 40 % formaldehyde sol. + 4·8 0·1 N NaOH
8·91   ,,   ,,   ,,   +   ,,   ,,   ,,   + 5·1   ,,
9·09   ,,   ,,   ,,   +   ,,   ,,   ,,   + 5·4   ,,

By a simple artifice the formation of polymethylimino-compounds in the above titrations may be prevented without the addition of sodium chloride solution. If, before adding the formaldehyde to the peptone solution some 0·1 N NaOH be added, then no cloudiness or precipitate forms. Repeating the above titrations in this manner and adding the solutions in the order named, the following results were obtained.

P_H^+
8·68   10 cc. Witte peptone soln. + 4·0 cc. 0·1 N NaOH + 10 cc. neutral 40 % formaldehyde +
                                                            0·80 cc. 0·1 N NaOH
8·91   ,,   ,,   ,,   + ,,   ,,   + 10 cc. neutral 40 % formaldehyde +
                                                            1·10 cc. 0·1 N NaOH
9·09   ,,   ,,   ,,   + ,,   ,,   + 10 cc. neutral 40 % formaldehyde +
                                                            1·40 cc. 0·1 N NaOH

In Fig. 3, the relation between the compositions of these mixtures and their P_H^+ values is represented diagrammatically.

The points obtained using phenolphthalein are marked +. The neutral red point is marked ⧻.
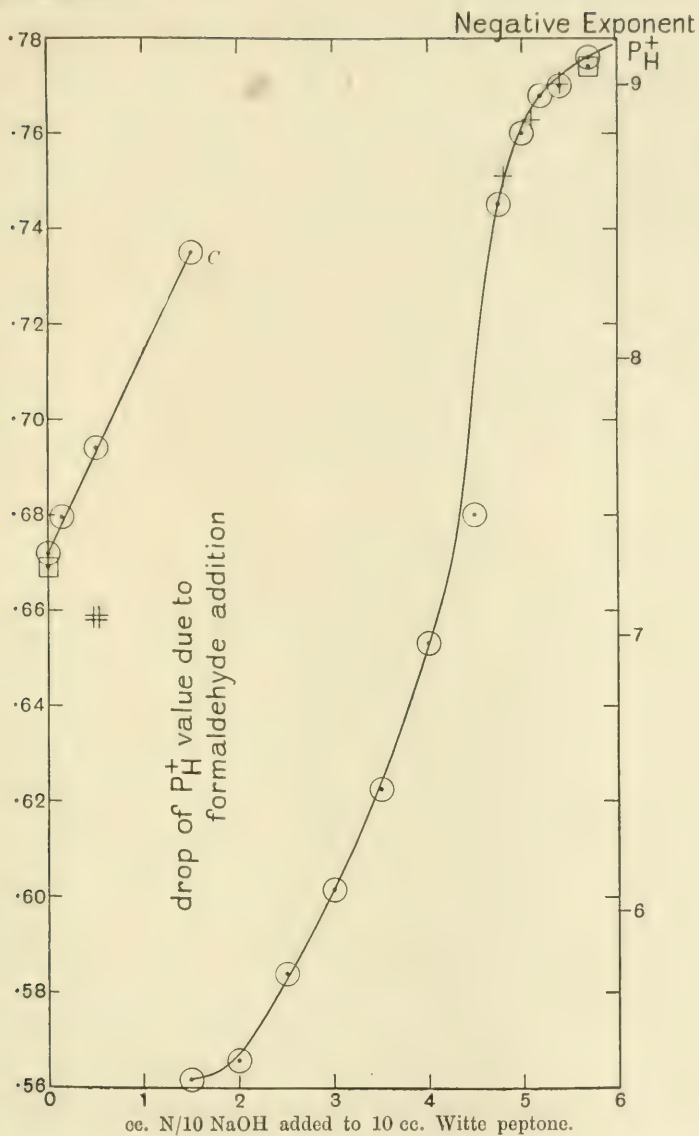
Fig. 3. Sörensen formaldehyde titration of 4 %/₀ Witte peptone performed electrometrically.

Note.—At *C* 10 cc. neutral formalin was added.
    ⊙ are points obtained in the titration process—electrode containing 0·3 cc.
    □ are points obtained with large electrodes in separate determinations.
    + are points obtained colorimetrically using phenolphthalein.
    ⧻ is point obtained colorimetrically using neutral red.

28—2

2. Sörensen titration of 4 % Witte peptone solution electrometrically.

10 cc. of peptone solution were taken and the same small electrode vessel as before. Connecting fluid and fluid in calomel half-electrode were saturated KCl solution; room temperature about 17°. As the alkaline formaldehyde solution becomes less alkaline on prolonged exposure to the air it is necessary to work with reasonable rapidity. After the addition of 1·5 cc. of 0·1 N NaOH solution the potential reading was found to be 0·7350; 10 cc. of neutral formaldehyde were then added and the potential immediately fell to 0·5615. Further additions of decinormal alkali were made—and corresponding potential readings taken—until after adding 4·5 cc. in all, the change of potential was so great, on account of the addition of the last 0·5 cc., that the ordinary rinsing out of the apparatus between two determinations was evidently not sufficient for this case, and the value 0·6800 is probably too low, the point being off the curve.

Experiments with solutions containing dissolved carbon dioxide.

The difficulties of H· determinations of fluids containing carbon dioxide have been so thoroughly dealt with by Michaelis and Rona [1909], Hassel-balch [1910], and Michaelis and Davidhoff [1912] that their repetition here is unnecessary. It is sufficient to remark that before a final unchanging potential reading can be obtained the hydrogen atmosphere, the fluid, and the platinum electrode must all be in equilibrium, no further gas exchange taking place between them. This refers to hydrogen, carbon dioxide, and oxygen. If the hydrogen be not at 760 mm. a correction must be applied to the formula expressing the relation between the potential of the half element and the hydrogen ion concentration of the fluid.

Generally speaking, electrode vessels may be divided into two classes, those in which hydrogen is passed in turn through a small portion of the fluid examined, and then through a portion of the same fluid in the cell itself until equilibrium is attained, and those in which one portion of hydrogen only is used. The methods are referred to respectively as those of the "moving" and "still" hydrogen atmosphere.

In electrode vessels in which a "still" atmosphere is employed it may be considered that there are three degrees of accuracy with which the H· concentration of a fluid containing carbon dioxide may be determined.

Firstly, the bubble of hydrogen may be brought to the surface of the fluid examined, the blackened platinum point saturated with hydrogen

adjusted so that it is just in contact with this surface, and the potential measured. The form of electrode used may be the V-electrode or the vertical electrode described. The potential will not represent accurately the reaction of the fluid. Owing to the diffusion of carbon dioxide from the fluid into the hydrogen the surface layers will rapidly become more alkaline than the solution was originally. The potential reading will therefore be high, falling slowly as equilibrium is established and only reaching a constant value some hours afterwards when that end has been attained.

Secondly, the small bubble of hydrogen may be passed backwards and forwards through the fluid in the cell a few hundred times before a reading is taken. By this means equilibrium is established, and it will be found that the value is constant and nearly correct. The electrode used may be a V-electrode manipulated in accordance with the instructions given by Michaelis [1912], or the vertical type filled by suction, or the Hasselbalch electrode. It will be seen that, though equilibrium is established between the hydrogen atmosphere and the fluid, the fluid has given up some carbon dioxide to the hydrogen and has therefore become more alkaline than it was originally and it is not the H˙ ion concentration of the original solution that has been measured.

This brings us to the third step where after equilibrium is reached the hydrogen bubble is retained, but the fluid in the cell replaced by a fresh volume with which the hydrogen is again brought into equilibrium. This process may of course be repeated until a definite final potential reading is observed which will then represent the true reaction of the fluid containing carbon dioxide.

The only electrode vessel described which permits of this is the Hasselbalch electrode vessel. Michaelis used it to control the results obtained by him using the V-electrode and passing the bubble up and down many times to obtain equilibrium—the second case above.

Since finding that the same thing can be done quite simply with the same electrode vessel as that described for gas-free solutions, I have made a number of determinations of the reactions of carbonate solutions by its means and have obtained consistent results.

Technique. If the electrode vessel be tipped sideways when filling, the liquid drawn up may be made to enter the side capillary leaving a bubble of hydrogen. After closing the tap the apparatus may be taken in the hand and by a slight movement at the wrist the bubble made to pass from one end of the vessel to the other as many times as are necessary to obtain equilibrium.

Bringing the vessel into a vertical position again the lower end is dipped below the surface of the fluid in the beaker, and the glass tube moved up or down through the rubber stopper until the platinum point just touches the surface of the column of fluid standing up in the electrode vessel. This may now be wiped dry externally, placed in the trough, and a reading of the potential taken. The value obtained corresponds exactly to that obtained by the V-tube used in the manner advocated by Michaelis. When small quantities only of carbon dioxide are present, this value will be very nearly correct. It may be checked by attaching a rubber tube to the tube at D (Fig. 1), introducing the lower end of the vessel in a sloping position into the beaker again, aspirating at D, and opening the tap gently. In this way fresh solution is drawn into B in the place of the old solution which passes out through D without disturbing the hydrogen bubble. This fresh quantity of solution is now brought into equilibrium with the bubble of hydrogen as before. The process may be repeated indefinitely and the result obtained is of the third order of accuracy—previously only obtained by the Hasselbalch electrode. The pattern of electrode vessel having a ground glass joint cannot be used for these operations as the height of the platinum point in the vessel is not then adjustable. It is essential that the platinum point shall only just touch the surface of the fluid when a reading is to be taken, otherwise equilibrium between the electrode and the solution, instead of taking only a minute or two, may take hours. In these cases, where the investigated liquid contains carbon dioxide, with experiments lasting over a number of hours, the rubber joint has a further disadvantage over and above that already mentioned. Carbon dioxide, like hydrogen, permeates rubber, and though the rubber joint is immersed in potassium chloride solution, transpiration of gases through the rubber is not prevented, and this slow transpiration is detrimental to accurate work when experiments last several hours.

Carbonate solutions. In order to check the results obtained when using the vertical electrode filled by suction for fluids containing carbon dioxide, I have determined the hydrogen ion concentration of mixtures of a sodium carbonate solution and dilute hydrochloric acid. For each determination 12·5 cc. of 0·2 N sodium carbonate were taken and diluted to nearly 100 cc. Then a measured quantity of 0·1 N hydrochloric acid was added and the volume made to 100 cc. exactly. No barometric correction has been applied to the determinations for diminished hydrogen pressure due to the carbon dioxide present. Neither have any steps been taken beyond the use of saturated potassium chloride as connecting solution to correct for diffusion potential. The results are plotted on a curve (Fig. 4). Abscissae represent

cc. of $0\cdot1$ N HCl taken: while ordinates are proportionate to the P_H^+ values less a constant. It will be seen that a mixture of $12\cdot5$ cc. of $0\cdot2$ N sodium carbonate + $12\cdot5$ cc. $0\cdot1$ N HCl diluted to 100 cc. corresponds to a solution of $0\cdot0125$ molecular NaHCO$_3$ which is also $0\cdot0125$ N with respect to sodium chloride. At this point the potential is $0\cdot7200$ against the calomel saturated-KCl half-electrode corresponding to $P_H^+ = 8\cdot13$. At this reaction phenolphthalein gives a pale pink colour, thus confirming the propriety of the analytical device of titrating carbonates in the presence of caustic alkali using phenolphthalein and methyl orange.

Potentiometer readings
against Calomel
Sat. KCl electrode
in volts

cc. $0\cdot1$ N HCl added to $12\cdot5$ cc. $0\cdot2$ N Na$_2$CO$_3$ solution.
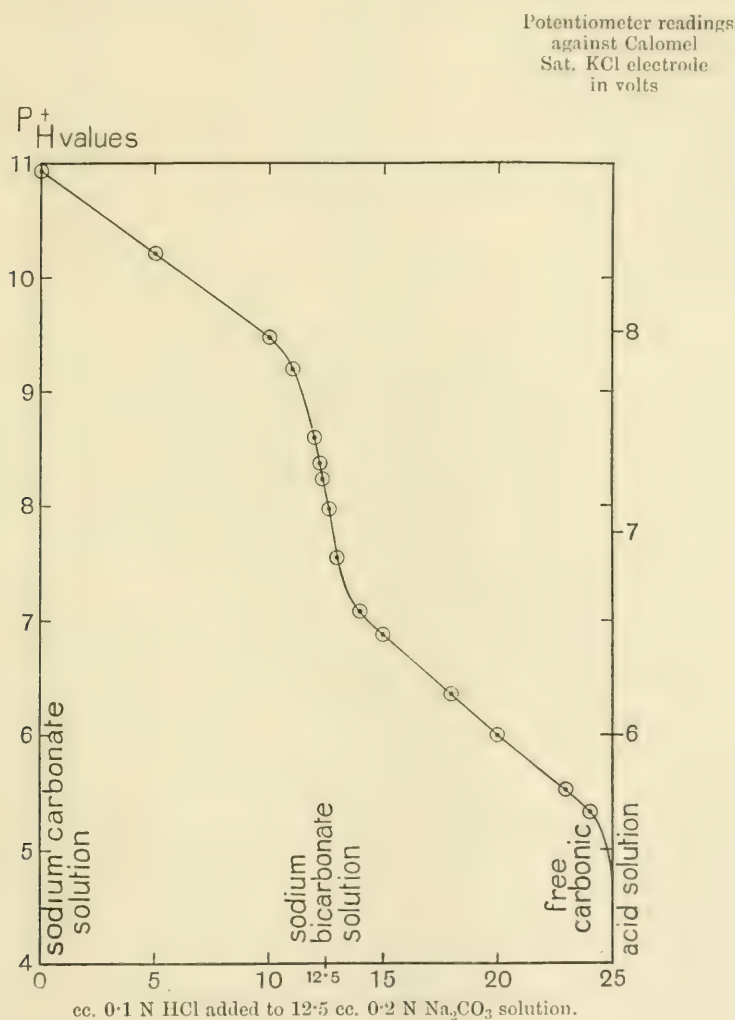
Fig. 4.

*Mixtures of 12·5 cc. 0·2 N Na₂CO₃ and varying quantities of 0·1 N HCl.
diluted to 250 cc.*

Connecting fluid sat. KCl. Half-electrode calomel-sat. KCl.

Vol. of 0·1 N HCl	Potential π	$P_H^+ = \dfrac{\pi - 0\cdot251}{0\cdot0577}$
0	0·883	10·95
5	0·841	10·22
10	0·798	9·48
11	0·782	9·20
12	0·755	8·73
12·3	0·738	8·44
12·5	0·720	8·13
12·7	0·701	7·80
13	0·684	7·50
14	0·652	6·95
15	0·638	6·71
18	0·618	6·36
20	0·598	6·01
23	0·570	5·53
24	0·558	5·32

REFERENCES.

Bjerrum (1910), *Zeitsch. physikal. Chem.* **73**, 724.

Cumming (1907), *Trans. Faraday Soc.* **11**, 1.

—— and Gilchrist (1913), *Trans. Faraday Society.*

Dolezalek (1899), *Zeitsch. Elektrochem.* **5**, 533.

Hasselbalch (1910), *Biochem. Zeitsch.* **30**, 317.

Michaelis and Rona (1909), *Biochem. Zeitsch.* **18**, 317.

—— and Davidhoff (1912), *Biochem. Zeitsch.* **46**, 131.

Sörensen (1909, 1), *Biochem. Zeitsch.* **21**, 167.

—— (1909, 2), *Biochem. Zeitsch.* **21**, 245.

Szili (1904), *Zeitsch. Elektrochem.* 113.

Wilsmore (1900), *Zeitsch. physikal. Chem.* **35**, 291.

XLII. SOME ESTERS OF PALMITIC ACID.

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The physiological importance and commercial value of the fats early attracted chemists to the study of their properties and composition. The interest which they then aroused has been fully maintained with the result that the present extensive literature on the compounds of the higher fatty acids deals almost entirely with the esters of glycerol to the exclusion of those of other polyhydric alcohols.

The chemistry of the fats may be said to have had its birth in the work of Chevreul [1823] to whose researches in the first quarter of the nineteenth century we owe the proof that the fats occurring in the animal body are triglycerides of higher fatty acids chiefly of stearic, palmitic and oleic. About forty years later Berthelot [1860] first succeeded in synthesising the mono-, di-, and tri-glycerides of many fatty acids and in identifying the tri-olein, tri-palmitin and tri-stearin so obtained with naturally occurring animal fats.

The method of synthesis adopted by Berthelot consisted in heating together glycerol and the fatty acid in a sealed tube to 200°–250°. This direct method of synthesis with various slight modifications has been largely used by later workers and still enjoys a wider application than the yields which are so obtained appear to warrant. Modifications of this method are chiefly directed towards eliminating the water formed during the reaction and thus enabling the process to be carried out at a lower temperature and also increasing the yield. A modification of this kind was introduced by Scheij [1899] and extended by Belucci and Manzetti [1911]. These workers found that the yields obtained by Berthelot's method could be greatly increased by carrying out the reaction under diminished pressure; thus Belucci and Manzetti synthesised triolein by heating together glycerol and oleic acid to 260° under a pressure of 20 mm., and state that in this way a yield of 95–98 %

was obtained; they afterwards found that the reaction could be carried out equally well under atmospheric pressure if a current of carbon dioxide were passed through the flask. Grün [1905] carried out the synthesis of diglycerides by warming the fatty acid and glycerol in concentrated sulphuric acid; thus a-a-distearin was obtained from glycerol and stearic acid, glycerol-disulphonic acid being formed as an intermediate product. None but primary alcohol groups are esterified in this reaction. Bloor [1910, 1912] applied this method to the condensation of mannitol with stearic and lauric acids; he identified his products as mannitol distearate and mannitol dilaurate respectively.

The halogen derivatives of glycerol have also been used in the preparation of glycerides. Partheil and Von Velsen [1900] prepared trilaurin and tripalmitin by heating the silver salts of these acids and tribromohydrin to 140° to 150° in the presence of xylene. A similar method was used by Guth [1903]; he prepared monostearin by heating together sodium stearate and monochlorohydrin, also a-distearin from a-dichlorohydrin, and β-distearin from β-dibromohydrin; tristearin was obtained from tribromohydrin and excess of sodium stearate. The yields obtained by these last two methods are not given.

Since the esters of the lower fatty acids are so readily prepared by the action of the acid chloride on the alcohol it seemed probable that this method might also be applied to the synthesis of esters of the higher fatty acids, not only with glycerol but also with other polyhydric alcohols. This method has been used recently by Grün and Schreyer [1912] who prepared a-myristo-a-chlorohydrin by the action of myristyl chloride on a-monochlorohydrin, and β-myristo-a-a-dichlorohydrin from myristyl chloride on a-a-dichlorohydrin. This is the only example of the use of this method which the writer has been able to find, though the wide use of the method in other fields makes it seem possible that some cases have been overlooked.

In order to test the efficiency of the above method for the synthesis of fatty bodies, palmityl chloride has been exclusively used and was prepared by the method described by Marie [1896] for melissyl and cerotyl chlorides. Its action was tried on ethylene glycol, glycerol, mannitol and glucose. In the case of glycerol esterification was effected by heating the two substances together to 120°. The action however was slow and this method was abandoned in favour of allowing the substances to react in the presence of pyridine with chloroform as a solvent. The action then took place in the cold and was completed by gently warming on the water bath.

EXPERIMENTAL.

Preparation of palmityl chloride. 45 g. of palmitic acid were mixed with 40 g. of phosphorus pentachloride (calculated amount 38 g.) in a distillation flask and the action started by warming. When the action was complete the excess of phosphorus pentachloride and the phosphorus oxychloride were distilled in vacuo on a boiling water bath; when these substances were removed the temperature was raised; the palmityl chloride distilled over as a colourless oil at 198°–200° under a pressure of 15 mm. Yield obtained 37 g. (77 %).

Preparation of glycol dipalmitate. 1 g. of dry glycol and 5 g. of pyridine were added to 12·8 g. of palmityl chloride (4 g. in excess of that required for complete esterification); about 50 cc. of dry chloroform were then added to dissolve the solid compound of pyridine and palmityl chloride formed; the contents of the flask turned a deep orange colour. The flask was closed and allowed to stand for 24 hours, it was then gently warmed on the water bath and well shaken; the chloroform was evaporated off and dilute sulphuric acid added to liberate the pyridine from its combination with palmityl chloride; the insoluble ester and palmitic acid (from excess of reagent) were filtered off and washed free from sulphuric acid and pyridine on a hot water filter; they were then dissolved in hot absolute alcohol and the free palmitic acid neutralised with alcoholic potash; the solution was evaporated to dryness and the residue transferred to a Soxhlet apparatus and extracted with ether. The ester crystallised from the ethereal solution on cooling in clusters of fine needles.

Yield before recrystallisation 7·5 g. (94 %). The substance so obtained was brownish yellow in colour and was soluble in hot and cold alcohol and also in chloroform, ether, ethyl acetate and the usual solvents for fats. It was recrystallised from a mixture of chloroform and alcohol since it was found that by this method the crystals obtained were white whereas when other solvents were used the colouring matter was thrown down with the crystals. The crystals so obtained were deposited in rosettes of fine needles; after four recrystallisations the substance had a constant m.p. of 65° (corrected). It was dried to constant weight in vacuo over sulphuric acid; it still, however, contained traces of moisture, which were only finally removed by heating in a toluene bath to 106°. The substance was identified by the results of elementary analysis and by the percentage of palmitic acid estimated by Hehner's method. As these values calculated for glycol monopalmitate,

glycol dipalmitate and for palmitic acid approach somewhat nearly to each other, these figures are appended for purposes of comparison.

	% Hydrogen	% Carbon	% Palmitic acid
Calculated for glycol monopalmitate	12·00	72·00	85·3
,, ,, glycol dipalmitate	12·27	75·84	95·2
,, ,, palmitic acid	12·5	75·01	100

Analysis:

(1) 0·2195 g.; 0·2406 g. H_2O; 0·6108 g. CO_2.
(2) 0·2598 g.; 0·2913 g. H_2O; 0·7227 g. CO_2.
(3) 1·8598 g.; 1·7750 g. palmitic acid.

	% Hydrogen	% Carbon	% Palmitic acid
Analysis (1)	12·44	75·9	—
,, (2)	12·45	75·84	—
,, (3)	—	—	95·5

The compound may therefore be identified as glycol dipalmitate corresponding to the formula

$$CH_2OOCC_{15}H_{31}$$
$$|$$
$$CH_2OOCC_{15}H_{31}.$$
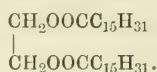
Glycerol tripalmitate (tripalmitin). 1·2 g. of glycerol and 9 g. of pyridine were added to 15·8 g. of palmityl chloride (4·7 g. in excess of that required for complete esterification); 30 cc. of chloroform were added. The procedure followed was the same as that described for the glycol compound. In this case the ester obtained from the ether extract was brown in colour and much difficulty was experienced in obtaining white crystals. After two recrystallisations from chloroform and alcohol followed by four from ether the substance, though melting sharply at 62°, was still biscuit coloured. It was then dissolved in wet ether and boiled for six hours with charcoal. The substance which crystallised from the ether after filtration was quite white and was deposited in fine needles; m.p. 62° (corrected). It was sparingly soluble in hot alcohol and soluble in ether, chloroform and all the usual solvents for fats. Traces of charcoal clung to the substance with great obstinacy and could not be removed by the filtration of the ethereal solution. They were finally removed by filtering the substance itself in the steam oven through a very small filter. The substance was dried at 110°, and identified by the results of elementary analysis; the corresponding values calculated for di- and tripalmitin and for palmitic acid are appended for comparison.

	% Hydrogen	% Carbon
Calculated for dipalmitin	11·90	74·06
,, ,, tripalmitin	11·91	75·95
,, ,, palmitic acid	12·5	75·01

Analysis:
(1) 0·2055 g.; 0·2222 g. H_2O; 0·5728 g. CO_2.
(2) 0·1915 g.; 0·2155 g. H_2O; 0·5405 g. CO_2.

	% Hydrogen	% Carbon
Analysis (1)	12·01	76·00
,, (2)	12·30	75·78

The substance was therefore identified as tripalmitin.

Owing to the difficulty experienced in purification sufficient substance was not available for a determination of palmitic acid.

Yield before recrystallisation 5·5 g. (50 %).

Yield of pure substance 2 g.

Mannitol hexapalmitate. 1·5 g. of mannitol and 8 g. of pyridine were added to 19·2 g. of palmityl chloride (4·8 g. in excess of that required for complete esterification). The procedure followed was identical with that described for glycol. The total yield of ester obtained after extraction in the Soxhlet apparatus and before further purification was 11·2 g. (78 %). The substance obtained was almost insoluble in cold alcohol, and very sparingly soluble in hot alcohol; it was easily soluble in ether, light petroleum, chloroform and ethyl acetate. It crystallised readily from a mixture of chloroform and alcohol or from ethyl acetate in rosettes of fine needles; m.p. 64·5 (corrected). It was dried at 106°.

The substance was identified by the results of elementary analysis and by the percentage of palmitic acid estimated by Hehner's method; these values calculated for mannitol hexapalmitate, mannitol pentapalmitate and for palmitic acid are also appended.

	% Hydrogen	% Carbon	% Palmitic acid
Calculated for mannitol hexapalmitate	12·05	76·03	95·4
,, ,, mannitol pentapalmitate	11·90	75·26	93·37
,, ,, palmitic acid	12·5	75·01	100

Analysis:
(1) 0·2245 g.; 0·2430 g. H_2O; 0·6270 g. CO_2.
(2) 0·2135 g.; 0·2335 g. H_2O; 0·5940 g. CO_2.
(3) 1·7067 g.; 1·6220 g. of palmitic acid.

	% Hydrogen	% Carbon	% Palmitic acid
Analysis (1)	12·03	76·19	—
,, (2)	12·14	75·90	—
,, (3)	—	—	95·06

The substance was therefore identified as mannitol hexapalmitate corresponding to the formula

$$C_{15}H_{31}CO.O.CH_2.(CH.OOCC_{15}H_{31})_4.CH_2.O.OCC_{15}H_{31}.$$
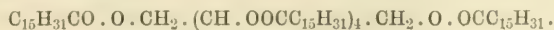
Glucose pentapalmitate. 1 g. of glucose and 9 g. of pyridine were added to 8·3 g. of palmityl chloride (2·4 g. in excess of that required for complete esterification). The procedure followed was the same as that previously described; as in the case of the glycerol compound decolorisation with charcoal and subsequent filtration of the melted ester were necessary. The total yield obtained after extraction in the Soxhlet apparatus was about 5 g. (83 %). The total yield of purified material was 2·8 g.

The substance obtained was very slightly soluble in cold alcohol and only sparingly soluble in hot alcohol; it dissolved readily in all the solvents commonly used for fats. It was recrystallised from ethyl acetate and melted at 62° (corrected). The substance was readily hydrolysed by alcoholic potash as was shown by the rapid darkening of the solution due to the action of the alkali on glucose, coupled with the fact that the substance was soon completely dissolved. The action of the glucose on the alkali prevented a saponification value from being taken. An attempt was made to estimate the palmitic acid by Hehner's method; a known weight of the substance was saponified with alcoholic potash, the alcohol evaporated off and the soap dissolved in water. The solution was very dark owing to the presence of the resins already mentioned, and it was found on acidifying and attempting to filter off the palmitic acid that the presence of these substances caused the palmitic acid to pass through the filter paper. An attempt to effect the complete acid hydrolysis and afterwards to estimate the glucose was next made; 0·397 g. of the ester was placed in a flask with 50 cc. of absolute alcohol in which, even on boiling, it only partially dissolved, the greater part sinking as oily drops; 25 cc. of 20 % hydrochloric acid were added and the flask boiled under a reflux condenser for 84 hours. At the end of this time the hydrolysis was still incomplete as was shown by the presence of small drops of insoluble ester in the boiling alcohol. When the contents of the flask were filtered to remove the unchanged ester and the filtrate neutralised the presence of glucose was shown by the reduction of Fehling's solution. Owing to the impossibility of removing the unchanged ester quantitatively the glucose could not be estimated. It was therefore necessary to rely solely on the combustion results for the identification of the substance.

$$0·2233 \text{ g.}; \quad 0·2410 \text{ g. } H_2O; \quad 0·6163 \text{ g. } CO_2.$$

	% Hydrogen	% Carbon
Analysis	11·99	75·26
Calculated for glucose pentapalmitate	11·82	75·32
„ „ „ tetrapalmitate	11·66	74·20

The substance was therefore identified as glucose pentapalmitate corresponding to the formula
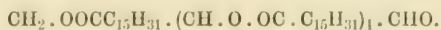
$$CH_2 . OOCC_{15}H_{31} . (CH . O . OC . C_{15}H_{31})_1 . CHO.$$

REFERENCES.

Belucci and Manzetti (1911), *Atti R. Accad. Lincei* [v] **20**, i, 125 and 235.
Berthelot (1860), *Chimie Organique fondée sur la Synthèse*, Vol. II.
Bloor (1910), *J. Biol. Chem.* **7**, 427.
—— (1912), *J. Biol. Chem.* **11**, 141 and 421.
Chevreul (1815–1823), *Recherches sur les corps gras d'origine animale.*
Grün (1905), *Ber.* **38**, 2284.
—— and Schreyer (1912), *Ber.* **45**, 3420.
Güth (1903), *Zeitsch. Biologie*, **44**, 78.
Marie (1896), *Bull. Soc. Chim.* **15**, 503.
Partheil and Von Velsen (1900), *Arch. Pharm.* **238**, 267.
Scheij (1899), *Rec. Trav. Chim.* **18**, 169.

XLIII. HYDROLYSIS OF PROTEINS WITH AN ALCOHOLIC SOLUTION OF HYDROGEN CHLORIDE.

PART I.

By CHARLES WEIZMANN and GANESH SAKHARAM AGASHE.

(Received June 30th, 1913.)

In the usual method of isolating the mono-amino-acids resulting from protein hydrolysis, aqueous hydrochloric acid is used as the hydrolysing agent, and subsequently a saturated alcoholic solution of hydrogen chloride is used for the purpose of esterification. An attempt was made to shorten this double process by using a saturated alcoholic solution of hydrogen chloride from the beginning to serve both as a hydrolysing and an esterifying agent.

Further, a saturated alcoholic solution of hydrogen chloride is undoubtedly a milder reagent than a saturated aqueous solution of the same, first, because the solubility of hydrogen chloride is smaller in alcohol than in water, and secondly, because it cannot be heated to as high a temperature as the aqueous solution ; so it was expected that the process of hydrolysis would not be quite complete, but might stop at an earlier stage than that of the amino-acids.

While the research was in progress, Pribram [1911] and Abderhalden and Hanslian [1912] published their investigations, in which the action of this reagent on proteins was studied. But their object being quite different from that of the present investigation, they have not pursued the subject further.

In the present investigation, experiments have so far been made on caseinogen from cow's milk, and on silk-fibroin. In each case it was found that the protein was only partially attacked. The corresponding mono-amino-acids were isolated by the usual methods, and converted into copper salts, which were analysed. It must be admitted that the evidence of these analyses is not absolutely conclusive ; but the authors believe that it is sufficient to show the identity of the various substances, which are known to be present in the different fractions by the more thorough investigations made by using the ordinary method.

·Although the proportion of the protein attacked could not always be exactly determined, as the reaction liquid could not be filtered free from the solid floating in it, it appeared certain that a very large proportion had been attacked. The yield of the amino-acids, however, was comparatively poor. This makes it extremely probable that a large proportion of products, more complex than the simple amino-acids, had been formed. The isolation of individual substances from this complex mixture is an extremely difficult task; and experiments are still being carried on for that purpose.

EXPERIMENTAL.

Caseinogen.

200 grams of caseinogen were hydrolysed by boiling for several hours with 2 litres of alcohol, saturated with hydrogen chloride. There was some unchanged solid which could not be filtered. The mixture was, therefore, evaporated in vacuo, and the residue decomposed with sodium hydroxide, and extracted with ether in the usual way. A very strong smell of ammonia was evident throughout this process.

The ethereal extract was then fractionated under 14 mm. pressure, and the following fractions collected. The temperatures are those of the bath.

$$\text{I } -60°, \text{ II } 60–100°, \text{ III } 100–140°, \text{ IV } 140–170°.$$

They were further worked up in the usual way.

Fraction I: This gave 0·4 gram of alanine, which was confirmed by analysing the copper salt.

0·0500 grm. gave 0·0162 grm. CuO. Cu = 25·92 per cent.
Calculated for alanine, Cu = 26·51 per cent.

Fraction II: This gave 3·5 grams of a mixture of leucine (also isoleucine), valine, and proline. They were isolated in the usual way, and confirmed by analysing the copper salts.

Leucine (and isoleucine):

0·1225 grm. gave 0·0293 grm. CuO. Cu = 19·13 per cent.
Calculated for leucine, Cu = 19·63 per cent.

Valine:

0·1098 grm. gave 0·0300 grm. CuO. Cu = 21·85 per cent.
Calculated for valine, Cu = 21·48 per cent.

Fraction III : This gave about 1·5 gram of a mixture of leucine, etc., and a little glutamic acid. The latter was converted into the copper salt, which was analysed.

0·0724 grm. gave 0·0280 grm. CuO.   Cu = 30·93 per cent.
Calculated for glutamic acid, Cu = 30·45 per cent.

Proline : About 1 gram of this was obtained from the last two fractions. It was confirmed by analysing the copper salt, which was first made anhydrous.

0·0572 grm. gave 0·0154 grm. CuO.   Cu = 21·53 per cent.
Calculated for proline, Cu = 21·77 per cent.

Fraction IV : Phenylalanine was isolated from this in the usual way, and confirmed by estimating the chlorine in the hydrochloride, of which about 0·4 gram was obtained.

0·1105 grm. gave 0·0778 grm. AgCl.   Cl = 17·38 per cent.
Calculated for phenylalanine hydrochloride, Cl = 17·61 per cent.

The rest of the fraction gave 0·5 gram of a mixture of solids.

The dark brown residue in the distilling flask was extracted with hot water. On evaporating the aqueous solution, about 10 grams of a yellowish white powder were obtained, which was most probably a mixture of diketo-piperazines.

The residue in the extraction flask was not worked up as usual for re-esterification, but is being worked up with the object of isolating the more complex products of hydrolysis.

Silk-fibroin.

50 grams of silk-fibroin were boiled for several hours with about a litre of alcohol, saturated with hydrogen chloride. The fibre was entirely broken down, but a very fine solid was still floating in the brown liquid, which, in this case, could be easily filtered off. The fine solid weighed about 14 grams on drying.

The liquid was further dealt with exactly as in the last case ; here, too, the smell of ammonia was very evident during the process of extraction with ether. The ethereal extract was distilled in the usual way, the temperature of the bath being only taken up to 100°. About 5·5 grams of a mixture of glycine and alanine were obtained. They were not separated quantitatively ; but the picrate of glycine was prepared according to Levene's method, and melted at 190°.

No crystalline solid could be obtained from the residue in the distilling flask, which, however, gave very beautifully Denigès' test for tyrosine, as modified by Mörner [1902].

The residue from the extraction is being worked up for the isolation of complex hydrolytic products, as in the last case.

Another interesting experiment was made with silk-fibroin. About a gram of it was heated with about 10 c.c. of absolute alcohol saturated with hydrogen chloride, in a sealed tube for about seven hours at 110–120°. The whole of the fibroin was found to have gone into solution without any residue.

REFERENCES.

Abderhalden and Hanslian (1912), *Zeitsch. physiol. Chem.* **77**, 285.
Mörner (1902), *Zeitsch. physiol. Chem.* **37**, 86.
Pribram (1911), *Zeitsch. physiol. Chem.* **71**, 472.

XLIV. THE FLOWER PIGMENTS OF *ANTIRRHINUM MAJUS*. 2. THE PALE YELLOW OR IVORY PIGMENT.

By MURIEL WHELDALE (*Fellow of Newnham College, Cambridge*) AND HAROLD LLEWELLYN BASSETT (*Trinity Hall, Cambridge*).

From the Laboratory of the John Innes Horticultural Institution and the Balfour Laboratory, Cambridge.

In a previous communication [Wheldale, 1913], the method of preparation of the crude pigment has been described. The varieties of *A. Majus* originally used for Mendelian experiments were the following:

> White
> Yellow
> Ivory
> Yellow tinged with bronze
> Ivory tinged with rose doré
> Bronze
> Rose doré
> Yellow tinged with crimson
> Ivory tinged with magenta
> Crimson
> Magenta.

Ivory is dominant to yellow and since white may carry any factor but the one producing yellow, the result of crossing any variety with white depends on the gametic constitution of the white individual used. Of the varieties forming anthocyanin, the Mendelian relationship may be represented in the scheme on p. 442. Dominance is denoted by the direction of the arrows: thus rose doré is dominant to bronze, magenta to rose doré, etc.

It has been previously suggested that ivory contains a chromogen of the nature of a flavone, from which the red and purple anthocyanins are formed by stages of oxidation or polymerisation or both. Also that the pigment

of the yellow variety and of the yellow patch on the palate of all varieties (except white) is due to a second, more deeply coloured flavone. Microscopic examination and microchemical tests show that anthocyanin and yellow pigments are mostly limited to the epidermis of the corolla, while the inner tissues contain the ivory chromogen. It is obvious therefore that all crude extracts of entire flowers will contain two or more pigments.
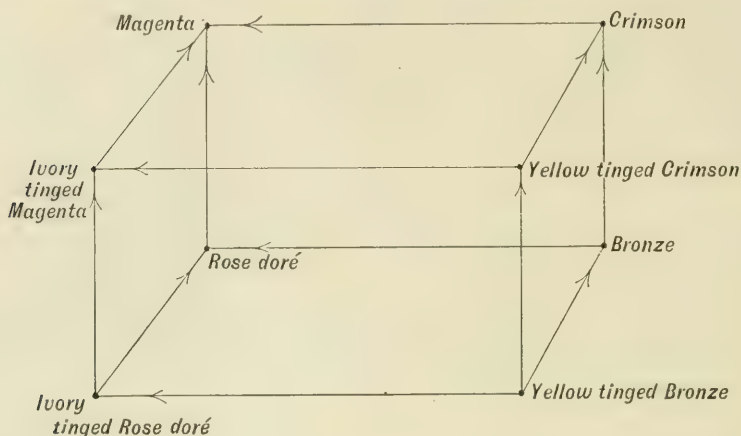
Although no analyses have yet been made of the anthocyanins from crimson and bronze, yet purification of the crude pigment indicates that these colours are merely due to mixture of magenta with yellow, and red with yellow respectively and not to specifically different pigments.

The constituent pigments of the varieties may be thus expressed:

Yellow (ivory, yellow).

Ivory, lower lip (ivory, yellow).

    ,,   upper lip (ivory).

Yellow tinged bronze, bronze, ivory tinged rose doré, rose doré (yellow, ivory and red).

Yellow tinged crimson, crimson, ivory tinged magenta (yellow, ivory, magenta).

Magenta, lower lip (yellow, ivory, magenta).

    ,,   upper lip (ivory, magenta).

The crude pigment from yellow, ivory (upper lip), ivory tinged magenta, bronze, rose doré, crimson and magenta (both upper and lower lips separately) was extracted with ether for several months.

The yellow ether extracts were in each case crystallised from alcohol, the first deposits, consisting mainly of the ivory pigment, separated off and the

melting points determined. All the products melted between 336° and 340°. In five cases the acetyl derivative was prepared by boiling with acetic anhydride as described in the previous paper and crystallising several times from ethyl acetate. The final products were white substances crystallising in needles and all melting at 181–182°.

The five acetyl derivatives were submitted to combustion, giving the following results:

Acetyl derivations of ivory pigment from

		C.	H.
(1)	yellow	63·10 %	3·94 %
(2)	ivory, upper lip	62·84 %	3·99 %
(3)	red	63·61 %	4·14 %
(4)	bronze	63·10 %	3·92 %
(5)	ivory, tinged magenta	63·23 %	3·88 %

which agree closely with combustion results of the acetyl derivative previously obtained from ivory extracted from magneta [Wheldale, 1913]:

Acetyl derivative of ivory pigment from magenta:

	C.	H.
(i)	63·19 %	4·30
(ii)	63·21 %	4·22
(iii)	62·95 %	4·31

It was there suggested that the ivory pigment might be apigenin, of which the acetyl derivative should theoretically give:

C.	H.
63·64 %	4·04 %.

An acetyl derivative of apigenin was obtained by Kostanecki [Rupe, 1909]. Perkin [1897], on the other hand, was unable to acetylate apigenin but prepared a benzoyl derivative by the Schotten-Baumann process. The derivative thus prepared was slightly soluble in alcohol, more readily in benzene, from which it crystallised in colourless needles melting at 210–212°.

A benzoyl derivative having the same properties and melting point as the above was prepared from the ivory pigment by the following method. The pigment was dissolved in about five times its weight of pyridine, cooled in ice and benzoyl chloride added. On addition of dilute sulphuric acid, a yellow oil separated out which was collected and spread on a porous plate. It solidified after a time and was extracted with ether which removed benzoic acid and some yellow products leaving a white wax-like substance. The latter was crystallised from benzene. Melting point 210–212°.

Analysis results:

Benzoyl derivative of ivory pigment:

C.	H.
73·82 %	4·02 %.

Theoretical result for benzoyl derivative of apigenin:

C.	H.
74·23 %	3·78 %.

The properties of apigenin given by Aberhalden [1911] are as follows:
Formula, $C_{15}H_{10}O_5$.
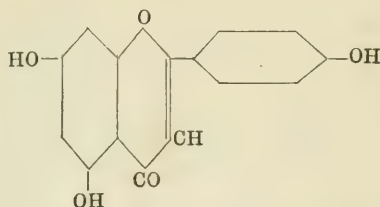
M. Pt. 347°.   Crystallises in yellowish-white platelets, readily soluble in alcohol, slightly in ether and hot water.   Becomes bright yellow in alkali solution.   The yellow solution in concentrated sulphuric acid has at first a greenish, later a bluish fluorescence.   The alcoholic solution gives a blackish-brown coloration with ferric chloride and brown-red with ferrous sulphate.

The ivory pigment corresponds entirely with this description.   From the acetyl derivative, the pure pigment was obtained by hydrolysis with alcoholic soda.   On neutralisation with acid, the pigment came down as a yellowish-white precipitate and after crystallisation from dilute alcohol, melted at 347°.

We therefore conclude that the ivory pigment is apigenin, and that it is present in each of the main classes of varieties of *Antirrhinum* with the exception of the white.   In the plant, apigenin exists undoubtedly as a glucoside, though the kind of sugar and the number of molecules attached still remain to be ascertained.

It appears possible that the deeper yellow pigment may prove to be a flavone, similar in constitution to apigenin, but deeper in colour owing to the presence of an additional hydroxyl group.

REFERENCES.

Abderhalden, E. (1911), *Biochemisches Handlexikon*, **6**, 51.
Perkin, A. G. (1897), *J. Chem. Soc.*, **71**, 801.
Rupe, H. (1909), *Die Chemie der naturlichen Farbstoffe*, **2**, 63.
Wheldale, M. (1913), *Biochem. J.* **7**, 87.

XLV. OBSERVATIONS ON THE USE OF THE FOLIN METHOD FOR THE ESTIMATION OF CREATINE AND CREATININE.

By WILLIAM HENRY THOMPSON,

THE LATE THOMAS ARTHUR WALLACE[1] AND

HAROLD REGINALD SEPTIMUS CLOTWORTHY.

From the School of Physiology, Trinity College, Dublin.

During a continuous use, for over three years, of the Folin colorimetric method of estimating creatine and creatinine we have made observations by way of testing its accuracy and limitations which we think may be useful to others if published. Some of these have meanwhile been pointed out by workers in other laboratories, but we include them for the sake of corroboration.

PROCEDURE.

The colorimeter we mostly used was that of Jobin—a modification of the Du Boscq instrument. We also compared its readings with those of two similar instruments made by Pellin[2] and with one of the Kagenaar pattern as described by Hoogenhuyze and Verploegh [1905]. We found that consistent and reliable readings were obtainable with all, after a little experience in their use had been acquired.

For making the standard bichromate solution we used Kahlbaum's purest bichromate of potassium certified to contain 99·97 % of the pure salt. This was fused, then cooled in a desiccator and a semi-normal solution made from it containing 25·54 g. in the litre.

The readings of the colorimeter were made by one of us (W. H. T.), but

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[1] Immediately before sending this paper for publication we learned, to our great sorrow, of the death of our co-worker, Thomas Arthur Wallace, at Agra in India, whither he had gone, a short while before, to take up a post in the Missionary College there. W. H. T., H. R. S. C.

[2] One of these was very kindly lent to us by Dr Ritchie of the Royal College of Physicians Laboratories, Edinburgh, to whom we express our best thanks.

were checked in all important cases by comparative readings made mostly by a second person in the laboratory familiar with the use of the instrument[1].

To avoid unconscious bias, the solutions in all the critical readings were made up by a second person and given to the reader under a number which gave no indication of which solution it was, this being disclosed only when the series was finished. We found this a very useful safeguard.

Great care was taken to obtain equal illumination of the two halves of the colorimeter. One important cause of inequality was found to be uneven blackening of the interior of the metal box in which the glass parallelopipeds are contained. The possibility of shifting of the zero on one or other side of the instrument was constantly examined and allowed for.

Another routine precaution, taken at the beginning of every set of readings and repeated as a rule after every four readings (if the series exceeded six in number), was to read the standard solution against itself. This we regard as of the utmost importance.

Few will believe, without actual experience, how much the reading of the standard by an individual may vary and how necessary is this control. The procedure also furnishes a ready test of fatigue, whether retinal or cerebral. When fresh, there is no difficulty in obtaining similar readings on the two sides using the standard solution in both tubes. When fatigued it was found that the readings were too high or too low, oftenest the latter and frequently to the extent of 0·5 mm.

The degree of illumination unquestionably affects the result. This has been pointed out by several workers. On dull days the readings tended to be lower than on bright days. Our instrument was placed opposite a large window, with northern aspect, and the readings made, for the most part, in the middle hours of the day. The practice of reading the standard against itself reduces the error for variation in the illumination.

CONTROL OF THE STANDARD SOLUTION.

Folin [1904], in fixing his bichromate standard, tested it against pure creatinine prepared from urine, also against creatinine solutions made by the conversion of creatine, and likewise against the double picrate of creatinine and potassium obtained from urine[2].

Of subsequent workers, there appear to be only three who controlled

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[1] We are grateful to Professor Noel Paton and Dr Rosenheim for assisting us in this way on more than one occasion.

[2] This last was overlooked by one of us (W. H. T.) in a recent note on the subject [1913].

their standard solutions by the first of these procedures, namely: Hoogen-huyze and Verploegh [1905], Koch [1905] and Klercker [1907].

The creatinine used by Hoogenhuyze and Verploegh was prepared from urine and gave almost theoretical readings (8·14 mm. instead of 8·1 mm.). That of Koch was obtained from Merck and required 11·46 mgms. to give the standard reading of 8·1 mm. in the colorimeter, this being normally given by 10 mgms. of creatinine treated in the way described by Folin. Klercker used a solution of creatinine prepared from urine. The content of this was determined by estimating the total nitrogen and found to be 96·6 % of the theoretical, assuming all the nitrogen to be present as creatinine.

We endeavoured to control our standards by this method, but early attempts to prepare pure creatinine did not prove satisfactory. Later we prepared a small sample of pure creatinine free from creatine. With this we obtained theoretical readings. The difficulty we encountered of pre-paring pure creatinine is admitted by Folin. This has been largely reduced by his newer methods.

Of these latter, that of heating pure crystalline creatine in the autoclave at a pressure of 4·5 kilos per sq. cm. for 3 hours was tried by one of us. The yield was not so high as claimed for it by Folin, nor the creatinine so pure as anticipated, but the method proved valuable in giving an easy means of preparing pure creatinine picrate which we used later as a control.

Most workers have tested their bichromate standard solution against solu-tions of creatinine prepared by the conversion of pure creatine. This was the method followed by Dorner [1907], Gottlieb and Stangassinger [1907], Hoogenhuyze and Verploegh [1908], Weber [1908], Benedict and Myers [1907]. We gave it an exhaustive trial. For this purpose a large number of samples of pure creatine, obtained from different kinds of flesh (horse, rabbit, dog), were prepared from time to time, and purified by recrystallising six to eight times till absolutely pure. Several Dumas analyses of these were made, the results of which are here shown.

TABLE I. *Showing results of Dumas analyses of samples of creatine.*

	Creatine taken	N. evolved	Temp.	Pressure	N. per cent.	Theory
1	0·1056	29·4 c.c.	16° C.	740 mm.	32·21	32·06
2	0·2220	61·8	18	740·8	32·03	,,
3	0·1625	44·0	17	755·5	31·92	,,
4	0·2020	54·8	16·5	756	32·22	,,
5	0·1260	35·4	22	753·4	32·10	,,
6	0·2160	58·0	18·8	754·6	32·30	,,

(Two of these estimates, Nos. 5 and 6, were kindly made for us by W. Caldwell, M.A., Senior Demonstrator of Physiology in this school, who

also checked readings for us at various times.   For this assistance we express to him our best thanks.)

There can be no doubt from the above analyses that our preparations of creatine were absolutely pure.   This is borne out also by the fact that the crystals in every case, after being fully air dried at room temperature, lost the calculated amount of water of crystallisation when further dried in the oven at 110° C.   In this latter drying—notwithstanding statements to the contrary—creatine does not decompose and we found it unsafe to trust for complete drying to a temperature lower than this.   In weighing the anhydrous creatine, which is very hygroscopic, great care was taken to guard against the absorption of water.

In making the test solutions of creatinine we usually took the quantity of creatine requisite to give when fully converted a 0·1 % solution of creatinine, that is to say, 0·116 grm. of anhydrous creatine or 0·132 grm. of crystalline creatine for 100 c.c.   In a few cases, solutions of anhydrous creatine of other strengths were used.

From a large number of estimates made with these solutions we found, as is shown in detail in Tables XIII and XIV, that the average conversion by the water bath method amounts to 96·5 % of the theoretical yield—varying from 93·1 % to 100·2 %.   The readings on the colorimeter scale corresponding to these were 8·39 mm. for the average, 8·7 mm. for the lower, and 8·08 mm. for the higher, instead of 8·1 mm. for the theoretical yield.

On more carefully examining the results published by others we found that our experiences were not singular.   Folin gives no data on this point in his first article, but speaks of the difficulty of complete conversion of creatine in the article in *Hammarsten's Festschrift* [1906].   Dorner [1907] with 0·1 % solutions of creatine obtained results varying from 85 to 100 % of the theoretical yield.   Gottlieb and Stangassinger [1907] 83·19 to 99·98 (with 4·56 % HCl) and 91·55 % to 92·98 % (with 2·28 % HCl).   Jaffé's [1906] best result with 2–2·5 % HCl was 94·3 %.   It did not seem to us therefore that this method was sufficiently accurate to be used as a control for the standard bichromate solution and another test was substituted by one of us the results of which have been published [Thompson 1913].   It was found that solutions of creatinine picrate and also of the double picrate of creatinine and potassium, when taken in quantities equivalent to 0·1 grm. creatinine and treated in the usual way, fulfilled the required conditions, giving readings of 8·1 mm. in the Du Boscq colorimeter.   This result settled some doubts which had previously existed in our minds with regard to the accuracy of the Folin standard.

Conditions which affect the Readings.

Our object was not to test all the possible conditions which might affect the results. We confined ourselves to those which came under our notice in the application of the method to our work.

These were (1) in the estimation of creatine, the best amount of acid (HCl) to use for the conversion into creatinine; (2) the optimum time and temperature for the development of the colour in the estimation of creatinine and creatine; (3) the influence of the quantity of alkali added for the same purpose; (4) the relative values of readings at different parts of the scale; (5) the influence of urinary pigment on the estimation of creatine in urine; (6) the estimation of creatine in the presence of dextrose and the use of phosphoric acid; (7) the recovery of creatine from diabetic urine; and (8) the reliability of the autoclave method.

1. *Estimation of Creatine. The amount of acid to be used in the conversion.*

For the conversion of creatine into creatinine, Folin in 1904 recommended 5 c.c. of normal HCl to be added to 10 c.c. of a dilute solution of creatine. Later [1906] he increased this amount to 10 c.c., a quantity which has been adopted by most subsequent workers. A few however, namely Dorner [1907], Hoogenhuyze and Verploegh [1905], have used double that quantity, namely 20 c.c. of N. HCl.

We have tested all three quantities and are convinced that the best results, both with the water bath and autoclave methods, are got by using 10 c.c. N. HCl with 10 c.c. of weak creatine solution. The conversion with 5 c.c. is liable to be less complete, particularly if glucose be present.

From several observations the following two consecutive sets of readings are taken:

N. HCl	Method	Reading	N. HCl	Method	Reading
20 c.c.	water bath	8·59 mm.	20 c.c.	autoclave	8·52 mm.
20	autoclave	8·69	10	,,	8·26
10	,,	8·46	5	,,	8·32
5	,,	8·6			

The readings with 20 c.c. and 5 c.c. are uniformly higher, that is, the yield is uniformly less, than with 10 c.c. It would seem as if a destruction of creatinine took place when 20 c.c. of acid were used.

Except at the very outset we have always used in these investigations 10 c.c. of N. HCl for the conversion of creatine.

·2.   *The Time and Temperature for Development of the Colour.*

Folin originally gave a time limit of 5 to 10 mins. for the development of the Jaffé reaction.   Later [1906] he restricts the time to 5 mins. and this period has been adopted by most workers.   Some however have departed from it, thus Dorner [1907] gave 5–15 mins., Benedict and Myers [1907] only 3·5 mins., Mellanby [1908] 5 mins. with some latitude, Mendel and Rose [1911] 10 mins.

Comparatively few observers however have recorded the temperature which they adopted.   Amongst these are Hoogenhuyze and Verploegh [1905], who diluted with water at 15° C., Dorner [1907], who cooled the solution after adding the picric acid and Klercker [1907], who used water for dilution at different seasons of the year of about the same temperature.   Mellanby [1908] also states that it is necessary for the temperature to be constant but does not give the degree.

We found with temperatures of 10° C. to 15° C. that the maximum colour was not developed under eight minutes.   Between 15° C. and 17° C. the time required was seven minutes, above 17° C. and below 20° C. five minutes is sufficient.

The results are shown in the following table:

TABLE II.   *Creatinine estimation: influence of time and temperature on the development of the Jaffé reaction.*

Readings Time 5 mins.	Readings Time 7 mins.	Temp.	Method of conversion
8·75 mm.	8·52 mm.	10	Water bath 5 hours.
9·30	8·85	10	Autoclave HCl 25 mins.
8·55	8·45	15	Water bath.
8·76	8·50	15	Autocl. HCl 25 mins.
8·80	8·70	15	Autocl. H_3PO_4 30 mins.
8·30	8·11	15	Water bath HCl.
8·48	8·35	17	Water bath HCl 3 hours.
8·29	8·30	20	Water bath HCl 3 hours.

Nearly all our observations were made at a temperature lying between 15° C.–17° C.   The flasks in which the reaction was carried out were weighted with leaden rings and kept in a basin of water at this temperature.

3.   *Effect of the amount of alkali used in the Folin method.*

We found that the quantity of alkali added to develop the colour of the Jaffé reaction distinctly affected the results.   For creatinine Folin recommended 5 c.c. of 10 % NaOH solution ; for creatine, the same amount over and above

that necessary to neutralise the acid used in the conversion. These quantities have been adhered to generally by others with few exceptions. Thus Benedict and Myers [1907] used 10 c.c. for 3·5 mins.; Mellanby [1908] concluded that no great accuracy was necessary; he got the same results with 10 c.c. and 3 c.c. His observations were chiefly concerned with meat extracts. Grindley and Woods [1906], also working with meat extracts, used 5 c.c. to 10 c.c. 10 % NaOH. Hehner [1907] soon afterwards stated, in a short publication giving no data, that an excessive quantity of alkali diminished the colour in the case of meat extracts, and that to obtain the best results the amount of alkali added must be quite small. He also considered that more picric acid than the quantity prescribed by Folin should be used when dealing with these extracts. In reply to this Emmett and Grindley [1907] repeated the work of Grindley and Woods, finding that in the determination of preformed creatinine the use of a small or large amount of alkali made almost no difference, but that slightly better results were obtained with 10 c.c. or 15 c.c. than with 5 c.c. For transformed creatine 10 c.c. and 15 c.c. gave the same results, and both were better than 5 c.c. It is not clear, however, that the acid used in the conversion was neutralised before adding the picric acid solution, nor was their method strictly comparable to that followed by others—the quantities of creatinine containing solution were much larger.

Cook [1909] also found in estimating the creatine content of meat extracts, that 5 c.c. of alkali did not give the maximum colour, while 10 c.c. and 15 c.c. gave identical results. With solutions of creatinine he found that 5 c.c. and 10 c.c. gave similar results. Accordingly, he recommended the use of 10 c.c. all round. For a considerable time we followed this recommendation without question, but later discovered that 10 c.c. of 10 % NaOH destroyed some of the creatinine and gave too low results. This applies chiefly to pure solutions of creatinine but also, though to a less extent, to urine. We have not tested the point in the case of meat extracts. Our results are shown in the following readings.

TABLE III. *Influence of the quantity of alkali on the colour developed.*

Readings with 5 c.c. NaOH of 10 % strength	Readings with 10 c.c. NaOH of 10 % strength
(a) Creatinine in aqueous solution	
8·29	8·75
8·25	8·70
8·58	8·75
8·78	9·05
8·20	8·58
Mean 8·42	Mean 8·77

TABLE III (*continued*)

(*b*) Creatinine in urine

9·54	9·66
9·05	9·45
9·55	9·75
6·30	6·50
7·51	7·9
8·25	8·27
6·26	6·41
5·73	5·85
Mean 7·77	Mean 7·97

It will be seen that with pure solutions of creatinine, the average reading of the series, when 5 c.c. of alkali were used, was 8·42 mm. (= 0·0966 % of creatinine): when 10 c.c. of alkali were used the average reading was 0·35 mm. higher, that is 8·77 (= 0·0924 % of creatinine). There was also a difference with urine though less marked. The mean of the series of readings with 5 c.c. of alkali is 7·77 mm. (= 0·1042 %), that with 10 c.c. alkali is 7·97 mm. (= 0·1016 % creatinine). So far as these solutions are concerned we are unable to confirm Cook: on the contrary, we find that better results are given when Folin's original directions are strictly followed.

4.   *Relative values of readings at different parts of the scale.*

It has been generally accepted that within a lower limit of 5 mm. and an upper one of 12 mm., as stated by Folin, the values of the readings are proportional. We have not found this to be so, nor is it generally true in colorimetry that the dilution ratio of a solution is inversely proportional to the height of the column of coloured solution. Folin natu ally refers to this, and explains the exceptional result in his method as follows. He concludes that dilution produces a diminution of the total colour of the solution—not an increase such as would arise if the colour were due to a red ion—but that the diminution is hidden by the increased relative depth of tint which occurs with increase of the height of column seen through. It has, however, since been shown by Chapman [1909] that the colour in the Jaffé reaction is due not alone to the formation of picramic acid, as had long been held, but to a mixture of picramic acid and a still redder reduction product of picric acid, namely diaminonitrophenol. It is doubtful therefore if Folin's explanation will hold good, nor are the facts quite in accordance with his view.

On this point we made a large number of observations, comparing in each case the reading given at a certain dilution with that given by the same solution diluted to one and a quarter, one and a half, or double the volume.

In the following table we give some of our results confining these to a comparison of readings with dilutions to 250 c.c. and 500 c.c., but making the selection so as to show the results with different solvents. These latter were water, dog's urine, normal human urine, and diabetic human urine.

TABLE IV. *Relative value of readings at different parts of colorimeter scale.*

	Solvent	Readings Dilution 10–250	Readings Dilution 10–500	Ratio	
1.	Water (HCl)	6·3 mm.	11·2 mm.	1 : 1·75	
2.	,,	6·5	11·36	1 : 1·72	
3.	,,	6·55	11·60	1 : 1·77	
4.	,,	5·50	10·10	1 : 1·80	
5.	,,	5·45	9·80	1 : 1·77	
6.	,,	6·15	11·00	1 : 1·79	Mean = 1 : 1·76
7.	,,	6·28	11·00	1 : 1·75	
8.	,,	6·15	10·90	1 : 1·77	
9.	,,	6·15	10·70	1 : 1·74	
10.	,,	6·30	10·90	1 : 1·73	
11.	,,	6·24	11·15	1 : 1·78	
12.	Dog's urine (HCl)	4·80	8·90	1 : 1·85	
13.	,, ,,	4·43	8·50	1 : 1·92	
14.	,, ,,	5·90	11·30	1 : 1·91	
15.	,, ,,	4·85	9·30	1 : 1·92	Mean = 1 : 1·91
16.	,, ,,	4·63	9·00	1 : 1·94	
17.	,, ,,	4·60	8·96	1 : 1·95	
18.	,, ,,	4·87	9·24	1 : 1·90	
19.	Human urine (HCl)	6·30	12·20	1 : 1·94	
20.	,, ,,	4·30	8·10	1 : 1·88	Mean = 1 : 1·91
21.	,, ,,	5·55	10·30	1 : 1·87	
22.	,, ,,	5·50	10·67	1 : 1·94	
23.	Diabetic urine	5·22	10·10	1 : 1·93	
24.	,, ,,	6·60	12·80	1 : 1·94	Mean = 1 : 1·87
25.	,, ,,	6·60	12·00	1 : 1·82	
26.	,, ,,	6·25	11·30	1 : 1·81	

On examining the list it will be seen that the higher reading is never double the lower and that the ratio differs considerably when urine is compared with water as the solvent, also that there is a difference, though not marked, between normal and diabetic human urine. On the other hand, the dilution ratio is identical for human and dog's urine. The mean ratio instead of being 1 to 2, is, for solutions in water 1 to 1·76, in dog's urine and human urine 1 to 1·91, in diabetic urine 1 to 1·87. These results were borne out by dilutions to other degrees than one to two.

Our general conclusions are that for accurate work readings can only be regarded as strictly proportional if they lie between a lower limit of 7 mm. and an upper one of 9 mm. If separated by a wider interval a correction factor has to be applied, and this varies for different solvents, but for all

practical physiological purposes may be based on the mean of all the above ratios for a difference of column of 5 mm., viz. $1:1\cdot8$ instead of $1:2$. In general therefore the upper readings of the scale give relatively too high results, the lower readings on the contrary too low results, as compared with the normal reading of $8\cdot1$ mm.

5. The influence of urinary pigment: recovery of creatine from normal urine.

Several observers have called attention to the influence of urinary pigment. Weber [1908] found that the darkening which occurs on boiling urine with HCl lowers the reading and increases the estimation of creatine by fully $5\,^0/_0$. Benedict [1912] also found a similar effect and recommended the use of granulated zinc in the boiling to remedy it.   Rose [1912] did not find this procedure efficacious with diabetic urines and recommended the use of phosphoric acid instead of hydrochloric.   Dreibholz [1908], with diabetic urine, found a difficulty in matching the colour with the standard bichromate solution after boiling the urine with hydrochloric acid, but failed to find a useful remedy.   With normal urine when the pigment was removed by filtration the result was not affected.

Early in these investigations it was clear to us that in the case of dog's urine the darkening on boiling with hydrochloric acid lowered the colorimeter readings.   We therefore proceeded to investigate the point, and did so in two ways, both of which were applied to normal human and dog's urine.

In the first method the ordinary creatine readings of a series of urines were compared with those given by similar samples of the same urines after boiling with an equal quantity of normal HCl.   In the boiling we used not alone the water bath for three hours, but also the autoclave at $117°–120°$ C. for two different periods, namely, 15 mins. and 25–30 mins.   The results are shown in the following tables:

TABLE V. *Showing colorimeter readings of human urine before and after boiling with normal HCl.*

	Unboiled	Water bath 3 hours	Autoclave 15 mins.	Autoclave 25–30 mins.
1.	7·8	7·53	7·5	7·6
2.	6·5	6·3	6·5	6·51
3.	8·1	7·9	8·15	8·1
4.	7·24	7·15	7·16	7·26
5.	7·6	7·3	—	7·5
6.	6·25	6·16	—	6·1
7.	7·51	7·54	—	7·5
Mean	7·29	7·13	7·19	7·22

The effect on human urine is therefore very slight. Taking the mean yield of the unboiled urines as 100 %, that of the water bath series amounts to 102·25 %, and of the autoclave, 25–30 mins., to 101 %. The yield of those boiled in the autoclave for 15 mins., when compared with the same group of urines unboiled, works out at 101·1 %.

TABLE VI. *Showing similar readings to the above for dog's urine.*

	Unboiled	Water bath 3 hours	Autoclave 15 mins.	Autoclave 25–30 mins.
1.	6·2	5·7	6·35	6·06
2.	7·4	6·08	6·46	6·45
3.	7·1	6·4	6·84	6·73
4.	7·74	7·3	7·54	7·47
5.	6·62	6·2	—	6·67
6.	8·5	7·87	—	7·9
7.	9·3	8·03	—	8·13
Mean	7·55	6·8	6·8	7·06

The results for dog's urine, taking the mean of the unboiled series as 100 %, work out as 111 % for urine treated by the water bath method, and 106·9 % for the same boiled in the autoclave for 25–30 mins. The mean yield of the smaller autoclave series boiled for 15 mins. gives 104·6 % when compared with that of the same group unboiled. It is interesting to note that in both series the effect on the pigment is more pronounced in the water bath than in the autoclave method.

The second method consisted in comparing the recovery of creatine when dissolved in urine with that of the same substance dissolved in water. The procedure adopted in testing the recovery of creatine from urine was as follows. Normal urines were taken and known quantities of the pure samples of creatine we used throughout this research were dissolved in them—for the most part 0·116 g. in 100 c.c. (= 0·1 % of creatinine).

Determinations were then made, (1) of the preformed creatinine in the urines unboiled, (2) of the readings of the same urines without any addition of creatine but treated by the water bath and autoclave methods, (3) of the total creatinine in the urines after creatine was added, these being treated as in (2). Two sets of subtractions were then made; (a) the creatinine of the unboiled urine was subtracted from the total creatinine of the urine to which creatine was added; (b) the creatinine of the boiled urine was subtracted from that of the same urine plus creatine.

The values for the first set of deductions should give too high results for recovered creatine, at all events in the dog's urine, if the darkening of the pigment affect the recovery. Those of the second set, presumably the

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true creatine values, should correspond to the results of recovery of creatine from solution in water provided no other disturbing factor, than the effect of the pigment, entered into the reaction.

Observations were made both with human urine and with that of the dog. The following tables show the results:

TABLE VII. *Showing the recovery of creatine from dog's urine: expressed as volume percentages of the urine taken.*

	Creatine added expressed as creatinine	Creatine recovered expressed as creatinine water bath 3 hrs.		Creatine recovered expressed as creatinine autoclave 25 mins.	
		(a)	(b)	(a)	(b)
1.	0·05	0·0627	0·0598	0·0575	0·0567
2.	0·05	0·0594	0·0475	0·0613	0·0533
3.	0·10	0·1003	0·1003	0·0916	0·0910
4.	0·10	0·1029	0·0935	0·0939	0·0892
5.	0·10	0·0892	0·0809	0·0925	0·0930
6.	0·10	0·1297	0·1221	0·1022	0·0950
7.	0·05	0·0598	0·0461	0·0605	0·0478
Totals	0·5500	0·6040	0·5502	0·5595	0·5260
		= 109·8 %	= 100 %	= 101·7 %	= 95·4 %

The total yield of the first set of values by the water bath method, column (a), works out as 109·8 % of the amount added. That obtained by the second method of deducting, column (b), gives a yield of 100 %. The corresponding values furnished by the autoclave method (25 mins. at 117–120° C.) are 101·7 % and 95·4 % respectively. Those therefore in column (a) show an increase due to pigment of 10 % by the water bath method and of 6–7 % by the autoclave method, figures which correspond very closely with the effects of darkening of the pigment of dog's urine seen in Table VI. Moreover, the mean of the recovery by the two methods, water bath and autoclave, obtained by the second method of deduction is 97·7 % which is very close to the average recovery from solution in water mentioned in the earlier part of this paper, namely, 96·5 %.

The results of recovery from human urine are seen in Table VIII.

On examining these figures it will be seen that the results of column (a) in the water bath method, which include the pigment effect, are just over 2 % higher than those of column (b). Similarly, those of the autoclave method show a difference of 1·2 % due to the pigment, and these differences correspond almost exactly with those given earlier for the effect of the pigment in human urine alone. The mean result for the two methods (water bath and autoclave), taking column (b) in each case, which excludes

the pigment effect, is 98·26 %, that is slightly higher than the general average for aqueous solutions. It may be concluded therefore that the darkening of the pigment is the only disturbing factor in normal urine in the recovery of creatine, and that its augmenting effect in round numbers is 2 % for human urine and 10 % for dog's urine. The effect, moreover, may be practically eliminated by the procedure of boiling the urine for the normal period (in any given research) and deducting the results from those given by the urine of subsequent periods treated in identically the same way— that is assuming the pigment has remained unaltered throughout.

TABLE VIII. *Showing the recovery of creatine from human urine: expressed as volume percentages of the urine taken.*

	Creatine added expressed as creatinine	Creatine recovered expressed as creatinine water bath 3 hrs.		Creatine recovered expressed as creatinine autoclave 25 mins.	
		(a)	(b)	(a)	(b)
1.	0·05	0·0521	0·0496	0·0563	0·0563
2.	0·10	0·0980	0·0970	0·0970	0·0974
3.	0·10	0·0980	0·0937	0·0958	0·0944
4.	0·10	0·1111	0·1092	0·1104	0·1072
5.	0·10	0·0881	0·0885	0·0922	0·0920
Totals	0·45	0·4473 = 99·4 %	0·4380 = 97·33 %	0·4517 = 100·4 %	0·4473 = 99·2 %

6. *Estimation of creatine in the presence of dextrose.*

Different statements had been made with regard to the influence of dextrose on the Jaffé reaction in its application to the estimation of creatinine and creatine. Klercker [1907] found that on long standing, when glucose was present, the colour became deeper; Hoogenhuyze and Verploegh [1905] that glucose produces no effect in the time necessary for the Folin estimation; Dreibholz [1908] that the caramel produced on boiling diabetic urine with HCl adversely affected the estimation of creatine; Taylor [1911] that glucose introduces no effect on the estimation of creatine unless present to above 5 %; while Rose [1912] strongly supports the view of Dreibholz.

We felt it necessary from the outset of our investigations to ascertain for our own satisfaction which statement was to be accepted. Our first observations dealt with aqueous solutions of creatine and dextrose, later we extended them to diabetic urine in which creatine was dissolved.

TABLE IX. *Estimation of creatine in presence of dextrose.*

	Water bath 3 hours HCl	Autoclave 25 mins. HCl	Autoclave 30 mins. H_3PO_4 3 %	Autoclave 30 mins. H_3PO_4 2 %	Dextrose present	Theoretical amt. expressed as creatinine
1.	0·1012	0·1020	—	—	5 %	0·1000
2.	0·1004	0·1002	—	—	10 %	,,
3.	0·0937	0·0936	0·0886	—	10 %	,,
4.	0·0910	0 0907	0·0814	—	10 %	,,
5.	0·0895	0·0900	0·0815	0·0900	10 %	0·0900
6.	0·0975	0·0968	0·0917	0·0960	10 %	0·1000
7.	0·0324	0·0366	0·0323	0·0326	10 %	0·0342
8.	0·0960	0·0964	0·0850	0·0889	10 %	0·1000
Av. % of recovery	96·6	96·6	86·7	92·5		0·1000

The foregoing table gives the first series of observations. It includes also a comparison of the results of the water bath method with those of the autoclave. Further, the efficacy of phosphoric acid in strengths of 3 % and 2 % for the recovery of creatine is compared with that of normal hydrochloric acid.

On examining the table it will be seen that with HCl—both water bath and autoclave methods—the average percentage of recovery (96·6 %) is practically the same as when dextrose is not present in the solution. This, as previously mentioned, was 96·5 %. Our conclusion, therefore, is that dextrose *per se* has very little, if any, influence on the results within the time necessary for estimating creatine.

In the above observations we used 10 c.c. of normal HCl for 10 c.c. of creatine solution, but in some of our early work the quantity of acid added was only 5 c.c. In these we found, when the quantity of sugar was 5 % or over, that the creatine results were much too low. This has also been the experience of M. Ross Taylor [1911]. It is essential therefore to use the larger quantity of acid.

It has been stated by Rose [1912] that the disturbing influence of dextrose is due to the action of hydrochloric acid in causing a transformation of some of the sugar into caramel. It occurred to him therefore to employ phosphoric acid in the conversion of creatine when dextrose is present, since it does not caramelise sugar. Rose claims to have obtained better results in this way than with hydrochloric acid. We tested the effects of the two acids, using solutions of creatine both in the presence and absence of sugar. In these tests varying strengths of phosphoric acid were used, 5 %, 4 %, 3 % and 2 %. The results are shown in Table IX. In all cases the best results were obtained by us with 2 %, by Rose, however, with 3 % phosphoric acid, the

volume of acid added in both sets of observations being 20 c.c. The results
with 5 % and 4 % phosphoric acid were much inferior—these strengths causing
a considerable destruction of creatinine.

We then felt it necessary to make a comparison of these same acids,
using creatine in aqueous solution. The following table gives the results:

TABLE X. *Estimation of creatine in aqueous solution: comparison of
hydrochloric and phosphoric acid in transforming creatine into creatinine.*

	Water bath N. HCl	Autoclave 30 mins. H_3PO_4 3 %	Autoclave 30 mins. H_3PO_4 2 %	Theoretical amt. expressed as creatinine
1.	0·0942	0·0910	0·0940	0·1000
2.	0·0935	0·0885	0·0920	,,
3.	0·0931	0·0915	0·0921	,,
4.	0·0928	0·0910	0·0931	,,
5.	0·0921	—	0·0925	,,
6.	0·0951	0·0932	0·0944	,,
7.	0·0976	0·0931	0·0976	,,
8.	0·0972	0·0944	0·0980	,,
9.	0·0960	0·0935	0·0942	,,
Mean recovery %	94·6	81·8	94·6	,,

On examining the table it will be seen that the results with normal
HCl and 2 % H_3PO_4 closely correspond, while those with 3 % H_3PO_4 are
considerably lower. The mean result with HCl is 94·6 % of the theoretical
yield; with 2 % phosphoric acid the same; with 3 % phosphoric acid 81·8 %.
It still remained to test the recovery of creatine from diabetic urine—the
results are shown in the following section.

7. *Recovery of creatine from diabetic urine.*

In these observations we made use of a series of diabetic urines obtained
from time to time from clinical hospitals in Dublin through the kindness of
members of the staff. We applied the same four methods for recovery
of creatine from diabetic urine which were used when the substance was
added to aqueous solutions of dextrose (see Table IX). The results are
necessarily somewhat more complicated than with simple solutions, since the
preformed creatinine and creatine had to be determined separately.

The full data are given in Table XI. Pure creatine was added to eight
samples of diabetic urine obtained from different patients. Three of the
samples contained both acetone and aceto-acetic acid, four were free from
both these bodies, and one contained a trace of aceto-acetic acid but no
acetone.

TABLE XI.

Showing the recovery of creatine when added to diabetic urines.

Urine contained	Preformed creatinine per cent.	Creatine added, expressed as creatinine	Preformed creatine of urine as found by				Creatine recovered as found by			
			(a) HCl W. B. method	(b) HCl autocl. 25 mins.	(c) H_3PO_4 3% autocl. 30 mins.	(d) H_3PO_4 2% autocl. 30 mins.	(a) HCl W. B.	(b) HCl autocl. 25 mins.	(c) H_3PO_4 3%	(d) H_3PO_4 2%
1. Dextrose 5·2 %. No acetone or aceto-acetic acid	0·0570	0·1000	0·0355	0·0346	0·0320	—	0·0948 =94·8 %	0·0950 =95 %	0·0945 =94·5 %	—
2. Dextrose 10 %. Both acetone and aceto-acet. ac.	0·0389	0·1000	0·0311	0·0311	0·0310	—	0·0720 =72 %	0·0720 =72 %	0·0611 =61·1 %	—
3. Dextrose 9·7 %. Both acetone and aceto-acet. ac.	0·0365	0·1000	0·0249	0·0319	0·0051	—	0·0859 =85·9 %	0·8660 =86·6 %	0·0812 =81·2 %	—
4. Dextrose 4·6 %. No acetone or aceto-acetic acid	0·0467	0·1000	0·0033	0·0089	0·0015	0·0040	0·0946 =94·6 %	0·0923 =92·3 %	0·0931 =93·1 %	0·0964 =96·4 %
5. Dextrose 3·8 %. No acetone or aceto-acetic acid	0·0675	0·1000	0·0128	0·0129	0·0104	0·0044	0·1065 =106·5 %	0·1053 =105·3 %	0·1001 =100·1 %	0·0950 =92·5 %
6. Dextrose 7·7 %. Both acetone and aceto-acet. ac.	0·0960	0·1000	0·0445	0·0441	0·0213	0·0267	0·0889 =88·9 %	0·0913 =91·3 %	0·0872 =87·2 %	0·0864 =86·4 %
7. Dextrose 2·8 %. No acetone, trace of aceto-acetic acid	0·0475	0·1000	0·0021	0·0022	0·0010	0·0000	0·0968 =96·8 %	0·0928 =92·8 %	0·0860 =86·0 %	0·0942 =94·2 %
8. Dextrose 1·7 %. No acetone or aceto-acetic acid	0·0374	0·0843	0·0268	0·0269	0·0196	0·0249	0·0804 =95·4 %	0·0786 =93·2 %	0·0798 =94·6 %	0·0749 =88·8 %

On examining the results it will be seen that the percentage recoveries with the different methods were as follows: (1) water bath HCl, 72 % to 106·5 %, the mean of the series being 91·8 %; (2) autoclave HCl, 72 % to 105·3 %, the mean being 91·2 %; (3) autoclave H_3PO_4 3 %, from 61 % to 100 %, the mean being 87·1 %; (4) autoclave H_3PO_4 2 %, from 86·4 % to 96·4 %, the mean being 91·76 %.

The results where HCl has been used are about 5 % inferior to the corresponding recoveries from aqueous and dextrose solutions. Those with 2 % H_3PO_4 are less variable but the mean (taken however from a smaller series) is identical with that of HCl. With 3 % H_3PO_4 the results are less favourable. We are forced therefore to conclude that while no special advantage is gained by using phosphoric acid, there is a liability to destruction of creatine by this reagent even with a strength of 3 %. It will be remembered that the same effect was found in the recovery of creatine from aqueous solutions both in the presence and absence of glucose.

The remarkable feature, however, of the results is the variability in the degree of recovery from different diabetic urines. This was not seen in the recovery from normal urine nor from aqueous or dextrose solutions, and points to a disturbing factor other than pigment or sugar. It is also remarkable that the lowest results were obtained from urines containing acetone and aceto-acetic acid. We have not studied the effects of either of these bodies, but they have been called attention to by Jaffé (acetone), Folin (acetone, aceto-acetic acid, and aceto-acetic ester), Klercker (acetone), Hoogenhuyze and Verploegh (acetone), Krause [1910] (acetone and aceto-acetic acid), Wolf and Österberg [1911] (aceto-acetic ester), Rose [1912] (aceto-acetic acid), Greenwald [1913] (acetone and aceto-acetic acid). Krause and Greenwald find the disturbing effect of aceto-acetic acid more serious than that of acetone. Its presence increased the values for preformed creatinine, and thereby lowered those of creatine. This is in harmony with our observations, but we think the matter deserves a more thorough investigation, starting with solutions of pure creatine to which dextrose, acetone, and aceto-acetic acid are added in succession.

Since the foregoing was written Graham and Poulton [1913] have published a note in which they show that aceto-acetic acid and its sodium salt diminish the colour of the Jaffé reaction and thus lower the estimate of creatinine. Aceto-acetic ester, used by Wolf and Osterberg, also by Folin, has no appreciable effect.

8. *Comparison of the autoclave and water bath methods.*

It may seem superfluous to have undertaken this part of the work, but at the period when we began it, the reliability of the autoclave method was not universally accepted. We therefore decided to test it for ourselves, and also to determine the optimum time in the autoclave for the transformation of pure creatine into creatinine at the temperature (117° C. to 120° C.) recommended by Benedict and Myers.

Two series of observations were made. In the first the results of heating on the water bath for three hours were compared with those of the autoclave, heated for 15 mins., 25 mins., and 35 mins. respectively. They are given in Table XIII:

TABLE XIII. *Creatine estimation : comparison of water bath and autoclave methods.*

No.	Water bath 3 hrs.	Autoclave 15 mins.	Autoclave 25 mins.	Autoclave 35 mins.	Theoretical amt. expressed as creatinine
1.	0·1002	0·0994	0·1004	0·0997	0·1000
2.	0·0981	0·0944	0·0982	—	,,
3.	0·0976	0·0935	0·0982	0·0942	,,
4.	0·0997	0·0942	0·0995	—	,,
5.	0·1000	0·0947	0·1000	0·1006	,,
6.	0·0944	0·0880	0·0962	0·0953	,,
Total	0·5900	0·5642	0·5925	0·3898	0·6000
	= 98·35 %	= 94·03 %	= 98·78 %	= 97·45 %	

It will be seen that the results of heating in the autoclave for 15 mins. are too low, while those for 25 mins. are identical, or almost so, with the water bath results. Consequently we are of opinion that the optimum time in the autoclave is 25 mins. at a temperature of 117° C. The results of heating for 35 mins. are slightly lower than those for 25 mins., indicating that the optimum period has been exceeded.

The second series gives a number of comparisons between the water bath results and those of the autoclave heated for one period only, namely, 25 mins.

On examining the mean results of the foregoing a remarkably close correspondence—indeed identity—will be seen, though the degree of conversion 95·7 % is a little lower than the average as given at the beginning of this paper, namely 96·5 %, which was obtained by taking the gross mean of the determinations in Tables XIII and XIV. When we compare this

with the gross mean of the autoclave determinations (25 mins.) the corre-
spondence also works out very close, namely 96·6 %, as against 96·5 %.
Observations were also made for periods of one hour and two hours in the
autoclave. In both cases there was a considerable destruction of creatinine,
the results at one hour giving a yield of 92·4 % and at two hours of 88·6 %.

TABLE XIV. *Creatine estimation: comparison of water bath
and autoclave methods.*

No.	Water bath 3 hours	Autoclave 25 mins.	Theoretical amount expressed as creatinine
7.	0·0975	0·0969	0·1000
8.	0·0964	0·0961	,,
9.	0·0964	0·0961	,,
10.	0·0994	0·0999	,,
11.	0·0994	0·0993	,,
12.	0·0942	0·0948	,,
13.	0·0935	0·0932	,,
14.	0·0931	0·0927	,,
15.	0·0931	0·0925	,,
16.	0·0944	0·0971	,,
17.	0·0959	0·0953	,,
18.	0·0953	0·0942	,,
19.	0·0960	0·0958	,,
20.	0·0955	0·0959	,,
Total	1·3401 = 95·7 %	1·3398 = 95·7 %	1·4000

It may be stated that we found no difficulty in obtaining consistent and
concordant results with the autoclave, provided that care was taken to see
that the time and temperature were kept constant and that the autoclave was
not opened till the pressure had fallen to zero. We allowed at least ten
minutes for this and then opened the valve slowly. Having set the auto-
clave for 117° C. it is desirable if possible not to use it for other purposes
necessitating different temperatures, during the time it is in use for estima-
ting creatine. It must be borne in mind that the velocity of reaction is
greatly accelerated as compared with that at 100° C. and therefore slight
differences of time and temperature produce much greater effects.

We may perhaps add also in regard to the water bath that our ordinary
rule was to immerse the flask in the water and use a reflux condenser.
Latterly we have returned to the use of the funnel and watch glass for
condensation and find it more convenient, but the flask should be immersed
in the water. If placed on the top of the water bath the results are less
consistent. Dr Rosenheim informed one of us (W. H. T.) that he had had
the same experience.

Summary of Results.

1. For control of the bichromate standard solution used in the Folin method of estimating creatinine and creatine we did not find the degree of conversion of creatine into creatinine by boiling with normal HCl sufficiently constant. We recommend for this purpose creatinine picrate as described by Thompson.

2. For the estimation of creatine in weak solutions the best results were got by using an equal quantity of N. HCl and boiling either on the water bath for three hours or in the autoclave for 25 minutes at 117° C.

3. For the development of the colour in the Folin method the optimum time and temperature in our hands proved to be 7 mins. at 15°–17° C.

4. The addition of too much alkali reduces the colour. For aqueous solutions and urine the best results are given by the quantity recommended by Folin, namely 5 c.c. of 10 % NaOH over and above what may be necessary to neutralise the solution.

5. The range of proportional readings on the colorimeter scale should not go lower than 7 mm. nor higher than 9 mm. If it is necessary to compare readings separated by a wider interval a correction factor should be used as explained in the body of this paper.

6. The darkening of the pigments of urine which occurs on boiling with normal HCl adds to the estimate of creatine-creatinine contained in it. With human urine the increase is slight ($1-2\frac{1}{2}$ %), with dog's urine as much as 10 %.

7. The presence of dextrose to the extent of 10 % did not affect the estimation of creatine. Phosphoric acid was not found to be superior to hydrochloric in estimating creatine when dextrose is present.

8. The recovery of creatine from diabetic urine gave results lower by 5 % than from aqueous or sugar solutions. Phosphoric acid did not prove more useful for this purpose than hydrochloric. The smaller recovery is probably due to the effect of aceto-acetic acid.

9. The autoclave method gave results identical with those of the water bath. The optimum time for urine and weak solutions of creatine was 25 mins. at 117°–120° C.

REFERENCES.

Benedict, F. G. and Myers, V. C. (1907), *Amer. J. Physiol.* **18**, 397.
Benedict, S. R. (1912), *J. Biol. Chem.* **12**, 73.
Chapman, A. C. (1909), *Analyst*, **34**, 475.
Cook, F. C. (1909), *J. Amer. Chem. Soc.* **31**, 673.

Dorner, G. (1907), *Zeitsch. physiol. Chem.* **52**, 225.

Dreibholz, W. (1908), *Inaug. Dissert.* (Greifswald).

Emmett, H. D. and Grindley, H. S. (1907), *J. Biol. Chem.* **3**, 491.

Folin, O. (1904), *Zeitsch. physiol. Chem.* **41**, 223.

— (1906), *Hammarsten's Festschrift*, "The Chemistry and Biochemistry of Kreatin and Kreatinin."

Gottlieb, H. and Stangassinger, R. (1907), *Zeitsch. physiol. Chem.* **52**, 1.

Graham, G. and Poulton E. P. (1913), *J. Physiol.* **46**, *Proceedings*, xliv.

Greenwald, I. (1913), *J. Biol. Chem.* **14**, 87.

Grindley, H. S. and Woods, H. S. (1906), *J. Biol. Chem.* **2**, 309.

Hehner, O. (1907), *Pharm. J.* **78**, 683.

Hoogenhuyze, C. J. van and Verploegh, H. (1905), *Zeitsch. physiol. Chem.* **46**, 415.

—— —— (1908), *Zeitsch. physiol. Chem.* **57**, 161.

Jaffé, M. (1906), *Zeitsch. physiol. Chem.* **48**, 430.

Klercker, K. O. (1907), *Biochem. Zeitsch.* **3**, 45.

Koch, W. (1905), *Amer. J. Physiol.* **15**, 15.

Krause, R. A. (1910), *Quart. J. Exp. Physiol.* **3**, 289.

Mellanby, E. (1908), *J. Physiol.* **36**, 447.

Mendel, L. B. and Rose, W. C. (1911), *J. Biol. Chem.* **10**, 213.

Rose, W. C. (1912), *J. Biol. Chem.* **12**, 73.

Taylor, M. Ross (1911), *Biochem. J.* **5**, 362.

Thompson, W. H. (1913), *J. Physiol.* **46**, *Proceedings*, i.

Weber, S. (1908), *Arch. expt. Path. Pharm.* **58**, 93.

Wolf, Ch. G. L. and Österberg, E. (1911), *Amer. J. of Physiol.* **28**, 71.

XLVI. NOTE ON THE IODINE CONTENT OF FISH-THYROIDS.

By ALEXANDER T. CAMERON.

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The presence of iodine in the tissues of, and oils from, various fishes has been known for a considerable time. It was found in herrings by Jonas [1838], in the oil from the liver of *Raia clavata* by Girardin [1842], and in crabs by Rieger [1853]. Barral [1877] states that iodine is always present in the oils from fish-livers. Stanford [1883] analysed a large number of fish oils, and invariably found iodine present. His analyses seem to have been controlled carefully, although the amounts quoted are minute. Thus:

Cod liver oil (various samples) averaged 0·000322 per cent. iodine (0·000138—0·000434).
Dry cod fish   contained 0·000829   ,,   ,,
Scotch herring, salted   ,,   0·00065   ,,   ,,
Scotch herring brine   ,,   0·00012   ,,   ,,
Seal oil   ,,   0·00005   ,,   ,,
Cod liver oil dragées (liver residues from
    which oil had been removed) contained 0·05637   ,,   ,,

Bourcet [1899], using analytical methods similar to those of Baumann, found iodine present in a large number of different species of fish, in amounts somewhat larger than those just quoted, ranging from 0·0007 % (*Leuciscus cephalus*) to 0·024 % (*Merlangus carbonarius*). *Raia clavata* contained 0·002 %. It is uncertain from his published data whether Bourcet's figures refer to fresh or dried fish.

Baumann and Goldmann [1896] fed dried cod to a dog as a probable source of iodine, and confirmed the presence of the element indirectly by noting that such feeding increased the iodine content of the thyroid.

So far as I am aware, fish thyroids have not hitherto been tested for iodine, although in them, as in mammals, its presence is to be expected, and in larger amount than in other tissue. Some indirect evidence on the normal presence of iodine in the thyroids of fishes is afforded by the results of

Marine and Lenhart [1910], who found that the so-called thyroid carcinoma of brook-trout is severe endemic goitre, which can be cured by the administration of iodine as in mammals. This would seem to indicate that these thyroids function in the same way as mammalian thyroids, and hence should contain the same constituents.

While it is still to be proved that iodine is an indispensable constituent of the thyroid, there can be no doubt that it is almost invariably present. Even in those cases where absolutely negative results have been recorded (compare for example Roos [1899], who found that the thyroids of many carnivorous animals contained no trace of iodine), the method employed— that of Baumann and Roos [1895]—is inconclusive for minimal amounts, since it frequently yields too low figures, indicating that minute quantities might entirely escape detection. (See in this connection Seidell [1911].) The actual amount of iodine found varies considerably, and seems to depend largely, if not entirely, on the diet. Roos' results [1899] show that the thyroids of herbivorous animals, whose food contains small quantities of iodine, are richer in iodine than those of carnivorous animals, whose food contains little of the element. For further literature in this connection see Swale Vincent [1912].

There is direct experimental evidence that when iodine compounds are fed the iodine content of the thyroid is markedly increased. Thus Baumann [1896] found that the thyroids of dogs previously fed for some weeks on lean meat (iodine free) contained little or no iodine. That of a dog which had received sheep's thyroids (9 grams) 14 days before death contained 0·34 % (dried gland). Roos [1899] obtained a very similar result. While a large number of analyses showed that the usual iodine content in the dried thyroid of the dog averaged about 0·1 %, that of a dog to which potassium iodide had been administered for some time contained 0·35 %. Baumann and Goldmann [1896] removed the left thyroid of a dog. Its weight, fresh, was 1·92 grams, and the iodine content 0·06 milligram. After the wound had healed, the dog was fed for 14 days on 14 pounds of dried cod (see above). The right thyroid was then removed. It weighed 2·5 grams fresh, 0·8 gram dry, and contained 2·9 milligrams iodine (0·36 %).

It might therefore be expected, taking into consideration the constant presence of iodine in sea-water, that the thyroids of salt-water fishes would contain a maximum amount of iodine. If the conclusions of Gautier [1899] are correct, and the iodine in sea-water is largely present in organised matter, it should be the more capable of assimilation and transmission.

The results given below afford evidence that unusually large amounts are

constantly present in some species, and hence lend support to the view that *the iodine content of the thyroid is a function of the iodine in the diet.*

Through the kindness of the authorities we have been furnished by the Marine Biological Association, Plymouth, with samples of glands of two species of elasmobranchs, *Raia clavata* and *Scyllium canicula.* In all, six samples were received, each consisting of a large number of glands. These had been obtained during the early spring of this year, and stored in absolute alcohol during collection. The alcohol was subsequently evaporated, and the residue added to the glands. The weights of fresh glands given below are therefore only approximate.

The samples were dried in a steam-oven until constant weight was attained, and were analysed by Hunter's method [1910]. This has now been tested by a number of investigators (see for example Seidell [1911]), and is undoubtedly very accurate when appreciable quantities of iodine are present. (In testing for minimal quantities of iodine in other work I have not found this method completely satisfactory, and my results so far seem to support Kendall's conclusions [1912].)

In all, four blank determinations were carried out (one with each series of analyses) using powdered fibrin as organic material; each gave an absolutely negative result. A test of the accuracy of the method, with a known quantity of potassium iodide, gave the following result:

<div align="center">
Amount of iodine taken 0·000306 gram.
,,   ,,   found 0·000310   ,,
</div>

This is of a lower order of magnitude than the amounts shown below, and in these the error is probably less. The data for the thyroids are seen in the following table:

Species	Sample	Weight (grams)		Amount taken (gram)	Amount of iodine (gram)	Percentage of iodine (dry substance)
		Fresh	Dry			
Raia clavata	(1 & 2)	(10·5)	2·541	0·503	0·002208	0·439
				0·503	0·002198	0·437
						(Mean 0·438)
	(3)	(6·4)	1·053	0·513	0·001678	0·327
	(4)	(?)	1·294	0·544	0·001542	0·283
Scyllium canicula	(5)	(4·5)	0·987	0·516	0·003712	0·719
	(6)	(?)	4·393	0·504	0·005831	1·157
				0·524	0·006099	1·164
						(Mean 1·160)

All the thyroids in sample 5 except two were from male specimens and all those in sample 6 from female specimens.

The results may be contrasted with the *maximum* iodine content hitherto observed in different mammals. In all cases the figures indicate percentage iodine in the dried gland.

Maximum iodine content in thyroids.

Fish thyroids 1·160 per cent.

Dog	,,	0·692	,,	recorded by	Marine and Lenhart [1909, 2].
Human	,,	0·588	,,	,,	,,	Seidell [1911].
Stag	,,	0·54	,,	,,	,,	Blum [1899].
Pig	,,	0·531	,,	,,	,,	Seidell and Fenger [1913] (fat-free).
Sheep	,,	0·53	,,	,,	,,	Baumann and Roos [1895, 1].
Beef	,,	0·477	,,	,,	,,	Marine and Lenhart [1909, 1].
Goat	,,	0·28	,,	,,	,,	Blum [1899].

The *average* iodine content can probably be taken as from 30 to 50 % of these figures.

While the thyroids of *Raia clavata* only contain a quantity of iodine of the same order as these maxima, both samples of *Scyllium canicula* contain more, and one *much more iodine than any thyroid previously reported on*. This is the more remarkable since this figure is the average for a large number of glands, while several of the other maxima (including the next three highest figures) are for single individuals. The theory that the iodine content of the diet plays a considerable, if not the whole, rôle in determining that of the gland therefore receives strong support from these figures.

The different results from the two sexes in *Scyllium* are possibly accidental. No previous marked variation seems to have been noted.

I hope to examine other species shortly, and to contrast thyroid with other tissue in what seems most probably the optimum condition for maximal iodine content.

I wish to thank Professor Swale Vincent for his kind interest in this work.

REFERENCES.

Barral (1877), *Compt. rend.* **84**, 308.
Baumann (1896), *Zeitsch. physiol. Chem.* **22**, 1.
—— and Goldmann (1896), *Münch. med. Wochensch.* **43**, 1153.
—— and Roos (1895, 1), *Zeitsch. physiol. Chem.* **21**, 481.

Baumann and Roos (1895, 2), *Zeitsch. physiol. Chem.* **21**, 489.

Blum (1899), *Arch. ges. Physiol.* **77**, 70.

Bourcet (1899), *Compt. rend.* **128**, 1120.

Gautier (1899), *Compt. rend.* **128**, 1069; **129**, 9.

Girardin (1842), *Compt. rend.* **14**, 618.

Hunter (1910), *J. Biol. Chem.* **7**, 321.

Jonas (1838), *Annalen*, **26**, 346.

Kendall (1912), *J. Amer. Chem. Soc.* **34**, 904.

Marine and Lenhart (1909, 1), *Arch. Internal Med.* **3**, 66.

—— and Lenhart (1909, 2), *Arch. Internal Med.* **4**, 440.

—— and Lenhart (1910), *J. Exp. Med.* **12**, 311.

Rieger (1853), *Jahresber. Chemie*, 329.

Roos (1899), *Zeitsch. physiol. Chem.* **28**, 40.

Seidell (1911), *J. Biol. Chem.* **10**, 95.

—— and Fenger (1913), *J. Biol. Chem.* **13**, 517.

Stanford (1883), *Chem. News*, **48**, 233.

Vincent (1912), *Internal Secretion and the Ductless Glands*, p. 314 (Arnold, London).

XLVII. THE COMBINATIONS OF HAEMOGLOBIN WITH OXYGEN AND WITH CARBON MONOXIDE. I.

By ARCHIBALD VIVIAN HILL (*Fellow of Trinity College, Cambridge*).

From the Physiological Laboratory, Cambridge.

In a previous paper Barcroft and Hill [1910] gave evidence to show that in dialysed solution haemoglobin has its smallest possible molecular weight, contains, namely, one atom of iron. In a later communication Hill [1910] suggested that the dissociation curves of oxyhaemoglobin in the presence of salts and carbon dioxide can be calculated from the hypothesis that these bodies tend to aggregate the large haemoglobin molecules into larger molecules, which then combine with oxygen according to the equation

$$\mathrm{Hb}_n + n\mathrm{O}_2 \rightleftharpoons \mathrm{Hb}_n(\mathrm{O}_2)_n.$$
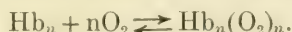
If y be the percentage saturation of the haemoglobin with O_2, and x the tension of the latter in the solution, it can be shown that this hypothesis leads to an equation for the dissociation curve of the type

$$y = 100 \, \frac{Kx^n}{1 + Kx^n} \quad \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots(1),$$

where K is the equilibrium constant and n is a whole number > 1.

This equation seems to suit all known dissociation curves of oxyhaemoglobin with a very high degree of accuracy, as numerous published and unpublished experiments of Barcroft [1913] and others (see e.g. Douglas, Haldane, J. S. and Haldane, J. B. S. [1912]) will show. In point of fact n does not turn out to be a whole number, but this is due simply to the fact that aggregation is not into one particular type of molecule, but rather into a whole series of different molecules: so that equation (1) is a rough mathematical expression for the sum of several similar quantities with n equal to 1, 2, 3, 4 and possibly higher integers.

The basis of the suggestion lies fundamentally in the idea that the

oxygenated haemoglobin molecules consist almost entirely of the fully saturated types

$$HbO_2, \ Hb_2(O_2)_2, \ Hb_3(O_2)_3, \text{ etc.,}$$
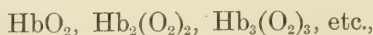
rather than of the partially saturated types Hb_2O_2, Hb_3O_2, Hb_4O_2, etc. For the observed dissociation curves in the presence of salts exhibit a double curvature (i.e. are of an **S**-shape), differing therein from the dissociation curve of dialysed haemoglobin, which latter is a rectangular hyperbola always concave to the horizontal axis. The former dissociation curves start from the origin almost—if not quite—horizontally, bend upward at first with concavity facing the y-axis, pass through a point of inflexion, and then bend inward again with concavity towards the x-axis. This main property of the curves is simply represented physically by the idea that the aggregated haemoglobin molecule, taken e.g. to be Hb_2, is oxidised into the form $Hb_2(O_2)_2$ by combination with two oxygen molecules simultaneously, and that the unsaturated molecule Hb_2O_2 either does not exist at all or exists in negligibly small quantities. For in the former case the dissociation curve is of the form $y = 100 \frac{Kx^2}{1+Kx^2}$, the tangent to which at the origin is $y = 0$, i.e. the horizontal axis: and this approximates to the fact observed experimentally. While if the unsaturated Hb_2O_2 exists in appreciable quantities the dissociation curve involves an equation of the type $y = B \frac{Kx}{1+Kx} + $ etc., where B is some constant less than 100, the tangent to which curve at the origin is $y = BKx$, a line going out at a slope as in the dissociation curve of dialysed haemoglobin, and not as observed when salts are present.

Now it seems at first sight almost unreasonable to suppose that Hb_2 would combine only with $2O_2$, i.e. with two molecules at once, and would not oxidise partially at first by combination with the single molecule O_2: that in fact the oxidation goes on according to the equation

$$Hb_2 + 2O_2 \rightleftarrows Hb_2(O_2)_2$$
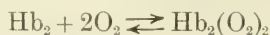
and not according to the equations

and then
$$\left. \begin{array}{l} Hb_2 + O_2 \rightleftarrows Hb_2O_2 \\ Hb_2O_2 + O_2 \rightleftarrows Hb_2(O_2)_2 \end{array} \right\} \ \dots\dots\dots\dots \text{ (A).}$$
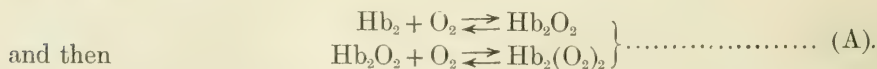
One could see no analogy for an equation of the former type when the latter scheme was so obviously possible. That the latter scheme does not immediately satisfy the facts is obvious from a short calculation, which however I believe will lead us to the clue to the whole matter. In equations (A) above, if K and K' be the equilibrium constants of the two reactions respectively, and

if the concentration of Hb_2O_2 be u, of $Hb_2(O_2)_2$ be v, of Hb_2 be w, and if x be the tension of oxygen and y the % saturation we find from the laws of mass-action

$$\frac{u}{wx} = K \text{ and } \frac{v}{ux} = K'.$$

Also since $(u + 2v)$ molecules of Hb are saturated with O_2, and there are $(2u + 2v + 2w)$ molecules of Hb altogether,

$$y = 100 \frac{u + 2v}{2u + 2v + 2w},$$

which becomes

$$y = 100 \frac{Kx + 2K'Kx^2}{2Kx + 2K'Kx^2 + 2}.$$

when we put

$$u = Kwx \text{ and } v = K'xu = KK'x^2w.$$

The tangent at the origin to this curve is

$$2y = 100Kx,$$

a straight line which is not the horizontal axis; thus the scheme represented by equations (A) does not give a dissociation curve of the observed kind— coming in horizontally to the origin—*unless K is exceedingly small.*

If however K is exceedingly small the difficulty entirely disappears, and the equation becomes

$$2y = 100 \frac{KK'x^2}{KK'x^2 + 1},$$

which is of the type required to fit the dissociation curve, with tangent at the origin $y = 0$. (It should be noted that if K is small K' must be large, so that KK' is finite.)

Can we therefore assume that K is small, that in fact oxygen combines far more readily with Hb_2O_2 than it does with Hb_2? In other words, is it a justifiable assumption that the partially saturated body Hb_2O_2 is very unstable, and exists in almost inappreciable quantities: that in fact Hb_2O_2 once formed almost immediately combines with another O_2 to form the saturated compound $Hb_2(O_2)_2$? In support of this contention[1] one may argue that there is no transitional spectrum corresponding to unsaturated oxides, and therefore that, if there is any truth in the aggregation theory, the unsaturated oxides are present only in negligible quantities. The theory will obviously lead directly to a dissociation curve of the observed type, to which by adjusting the value of KK' it can be made to conform with some degree of accuracy. So far then as we are concerned with dissociation curves of oxyhaemoglobin, the equations to these can be deduced at once from the

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[1] First made to Mr Barcroft by Mr W. H. Mills of the Chemical Laboratory, Cambridge.

aggregation hypothesis with the conception of gradual combination with oxygen according to the scheme

$$Hb_n + O_2 \rightleftarrows Hb_nO_2,$$
$$Hb_nO_2 + O_2 \rightleftarrows Hb_n(O_2)_2,$$
$$Hb_n(O_2)_2 + O_2 \rightleftarrows Hb_n(O_2)_3, \text{ etc.,}$$
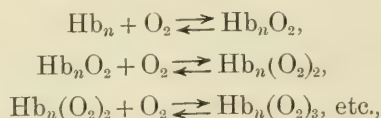
provided always that we may assume that O_2 combines much more readily with Hb_nO_2 to form $Hb_n(O_2)_2$, than with Hb_n to form Hb_nO_2: *in other words that the partially saturated molecule is very unstable, is difficult to form and easy to combine further with oxygen.* For all such combinations lead to a dissociation curve with double curvature, which reaches the origin horizontally.

A plausible suggestion as to the reason why O_2 should combine much more readily with Hb_2O_2 than with Hb_2 may be put forward here. The haemoglobin molecules are probably aggregated by surface effects, by the neutralisation e.g. of the electric charges on their surfaces. It may be however that the free chemical " bonds " of Hb, with which reduced haemoglobin attaches itself to oxygen, are in addition combined with one another in the aggregated molecule, so that if reduced haemoglobin is Hb= then the aggregated molecule is not merely Hb, Hb (two molecules just sticking together by surface forces) but rather Hb=Hb. In that case Hb_2O_2, the half saturated molecule, has to be formed by the breaking loose of these bonds, and will be =Hb, Hb=O_2. This body one would naturally expect to be very unstable, as is Hb= in the presence of oxygen. It would be very difficult for O_2 to form this compound, because it has to break down the Hb=Hb molecule into =Hb, Hb= in order to do it: and the compound =Hb, Hb=O_2 once formed, presenting as it does two unsaturated bonds, would immediately seize on another O_2 molecule becoming O_2=Hb, Hb=O_2. Whether this turns out to be the case or not, it presents a very clear physical conception as to why Hb_2O_2 should exist in only very minute concentrations.

So far then the aggregation hypothesis can be made to fit the observed facts very closely, and to have a reasonable physical explanation. Some doubt has been thrown on the particular form of it advanced in my earlier paper, by Douglas, Haldane and Haldane [1912]. These authors determined experimentally the CO-haemoglobin curve in the presence of a partial pressure of oxygen, and found it to be a rectangular hyperbola. According to the rough scheme, dealing with single molecules,

$$HbO_2 + CO \rightleftarrows HbCO + O_2,$$
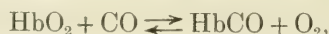
(and supposing the haemoglobin to be all combined with either CO or O_2) the equation to the CO-dissociation curve in the presence of a constant partial pressure of O_2 should be that of a rectangular hyperbola. But dealing with aggregated molecules, say Hb_2, the formula

$$Hb_2(O_2)_2 + 2CO \rightleftarrows Hb_2(CO)_2 + 2O_2$$

leads, by the laws of mass action, to an equation $\frac{y'x^2}{yx'^2} = K$, if x and x' be the

partial pressures of O_2 and CO, and y and y' be the % saturations with O_2 and CO. If all the haemoglobin is combined $y + y' = 100$, so that

$$\frac{y'}{100 - y'} = K\,\frac{x'^2}{x^2},$$

which represents a CO-dissociation curve of the type described above, containing a point of inflexion, and coming in horizontally to the origin. Thus this, and as a matter of fact all similar conceptions, of the balanced action between CO, O_2 and haemoglobin, led to the same type of equation, which gives a curve in no way like a rectangular hyperbola: so that the experiment of Douglas and Haldane led one to doubt the validity of the whole hypothesis of aggregation, in spite of the accuracy with which it fitted some of the facts. *Why is it that the CO-O_2-haemoglobin dissociation curves, in the presence of a constant tension of O_2, are unaffected by salts and CO_2, when the O_2-dissociation curves and the CO-dissociation curves are so very largely affected?* This very puzzling question can, as a matter of fact, be quite simply answered by the aid of the conception sketched above.

Let us assume for simplicity that the haemoglobin exists as double Hb_2 molecules, and that according to the following scheme it combines gradually and by steps with CO and O_2. The Hb_2 combines first with one O_2 or with one CO, the new molecule Hb_2O_2 or Hb_2CO combines further with O_2, or CO, to form one of the three compounds

$$Hb_2(O_2)_2,\ \ Hb_2(O_2)(CO),\ \text{ or }\ Hb_2(CO)_2.$$
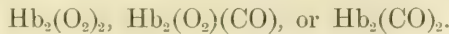
We then have the equations (in which the small letters represent concentrations and the k's represent equilibrium constants)

$$
\begin{aligned}
&\underset{y}{Hb_2} + \underset{x}{O_2} \ \rightleftarrows\ \underset{u}{Hb_2O_2} && k_1 \\
&\underset{u}{Hb_2O_2} + \underset{x}{O_2} \ \rightleftarrows\ \underset{v}{Hb_2(O_2)_2} && k_2 \\
&\underset{u}{Hb_2O_2} + \underset{x'}{CO} \ \rightleftarrows\ \underset{w}{Hb_2(O_2)(CO)} && k_3 \\
&\underset{y}{Hb_2} + \underset{x'}{CO} \ \rightleftarrows\ \underset{u'}{Hb_2CO} && k_1' \\
&\underset{u'}{Hb_2CO} + \underset{x'}{CO} \ \rightleftarrows\ \underset{v'}{Hb_2(CO)_2} && k_2' \\
&\underset{u'}{Hb_2CO} + \underset{x}{O_2} \ \rightleftarrows\ \underset{w}{Hb_2(CO)(O_2)} && k_3'
\end{aligned}
\qquad\Bigg\}\ \dots\dots\dots(A).
$$
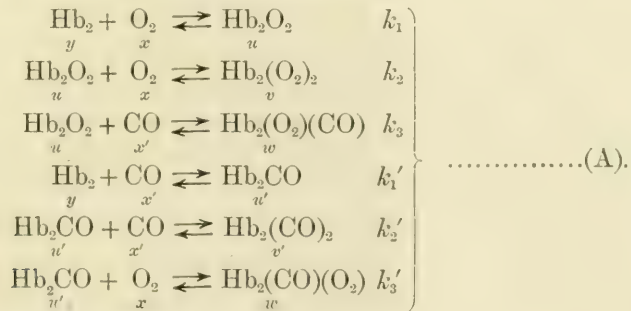
From these, by the laws of mass action, we find

$$
\text{I.}\qquad \frac{u}{xy} = k_1,\qquad \frac{u'}{x'y} = k_1',
$$

$$
\text{II.}\qquad \frac{v}{ux} = k_2,\qquad \frac{v'}{u'x'} = k_2',
$$

$$
\text{III.}\qquad \frac{w}{ux'} = k_3,\qquad \frac{w}{u'x} = k_3'.
$$

From equations I and II, multiplying up, we find

$$\text{IV.}\qquad \frac{v}{x^2y}=k_1k_2,\qquad \frac{v'}{x'^2y}=k_1'k_2',$$

and from equations I and III

$$\text{V.}\qquad \frac{w}{xx'y}=k_1k_3,\qquad \frac{w}{xx'y}=k_1'k_3',$$

so that $k_1k_3=k_1'k_3'=k$ say.

Let ξ be the amount of haemoglobin per c.c. saturated with O_2: ξ' the amount saturated with CO. Then

$$\xi=\tfrac{1}{2}(u+w)+v,$$
$$\xi'=\tfrac{1}{2}(u'+w)+v'.$$

Using the values of u, v and w given in equations I, IV and V we find

$$\left.\begin{aligned}\xi&=\tfrac{1}{2}(k_1xy+kxx'y)+k_1k_2x^2y\\ \xi'&=\tfrac{1}{2}(k_1'x'y+kxx'y)+k_1'k_2'x'^2y\end{aligned}\right\}\dots\dots\dots(B).$$

and

$$\therefore\quad \frac{\xi'}{\xi}=\frac{x'}{x}\times\frac{\tfrac{1}{2}(k_1'+kx)+k_1'k_2'x'}{\tfrac{1}{2}(k_1+kx')+k_1k_2x}.$$

Thus apparently the % saturations of the Hb with O_2 and CO are *not* in the direct ratio of the latter's tensions, as is necessary if the dissociation curve is to be a rectangular hyperbola. We come however to the hypothesis outlined above, viz. that the unsaturated Hb_2O_2 is very unstable and tends to break down either into Hb_2 or Hb_2O_4. In this case we may assume that k_1 is very small, and k_2 very large, their product remaining finite. Similarly we have assumed that k_1' is very small and k_2' is very large. We therefore find, neglecting k_1' and k_1,

$$\frac{\xi'}{\xi}=\frac{x'}{x}\frac{\tfrac{1}{2}kx+k_1'k_2'x'}{\tfrac{1}{2}kx'+k_1k_2x}\qquad\dots\dots\dots\dots\dots\dots(C).$$

This, again, is not a direct proportion between ξ'/ξ and x'/x, as we should expect from Douglas and Haldane's observations [1912, see especially pp. 278, 290]. Looking however at the chemical equations (A) above, if we were to assume that CO has a very much higher affinity for the partially saturated $=Hb, Hb = O_2$ (i.e. Hb_2O_2) than has O_2, i.e. that nearly all the Hb_2O_2 combines with CO rather than with O_2: and if we further assume that the partially saturated $=Hb, Hb = CO$ (i.e. Hb_2CO) has a much higher affinity for the CO than it has for the O_2, i.e. that nearly all the Hb_2CO combines with CO rather than with O_2, then the whole difficulty is immediately solved. For[1] in the first case we find that k_3x' is very much greater than k_2x, and

---

[1] It should be noted that the assumption is not simply that Hb_2O_2 has a greater affinity for a given pressure of CO than it has for the same pressure of O_2: this, of course, we should expect. It is that the affinity of Hb_2O_2 for CO exceeds the affinity of Hb_2O_2 for O_2 more than the affinity of Hb for CO exceeds the affinity of Hb for O_2: that the CO-affinity is even greater, relatively to the O_2-affinity, in the aggregated molecule than it is in the simple.

in the second that $k_2'x'$ is much greater than $k_3'x$. These assumptions are in the highest degree reasonable, and making them, equation (C) above immediately gives the required result. Putting $k = k_1'k_3'$ in the numerator and $k = k_1k_3$ in the denominator (as shown above) we find

$$\frac{\xi'}{\xi} = \frac{x'}{x} \frac{k_1'}{k_1} \frac{\frac{1}{2}k_3'x + k_2'x'}{\frac{1}{2}k_3x' + k_2x}$$

and assuming, as above, that $k_3'x$ can be neglected in comparison with $k_2'x'$, and that k_2x can be neglected in comparison with k_3x', we find

$$\frac{\xi'}{\xi} = \frac{x'}{x} \frac{k_1'}{k_1} \frac{k_2'x'}{\frac{1}{2}k_3x'}$$

$$= \frac{x'}{x} \frac{2k_1'k_2'}{k_1k_3}.$$

That is to say, the CO- and O_2-saturations of the haemoglobin are proportional to their partial pressures, and if the haemoglobin be fully saturated with CO and O_2, i.e. putting $\xi + \xi'$ equal to a constant (say 100) we find, if $2k_1'k_2'/(k_1k_3) = K$,

$$\frac{\xi'}{100 - \xi} = \frac{x'}{x} \times K.$$

This is a rectangular hyperbola between ξ', the % saturation with CO, and x', the partial pressure of the CO. Thus on quite simple assumptions the theory is found to agree perfectly with the experimental observations of Douglas and Haldane.

Now Douglas and Haldane have brought to light some other experimental facts which seem at first sight to be difficult of explanation. They found [1912, p. 279] that the CO-O_2-haemoglobin dissociation curve, in the presence of a fixed tension of CO and a variable tension of O_2, was a rectangular hyperbola for large tensions of oxygen but bent back and fell again as the O_2-tension became very small and gradually vanished. Now, on our theory as advanced above, a very pretty explanation of this fact can be found, which may elicit some facts of further physiological interest. The assumption made above is practically that CO combines much more readily with Hb_2O_2 in the presence of O_2, than it does with Hb_2 in the presence of the same O_2. When the O_2-pressure is reduced beyond a certain limit very little Hb_2O_2 is formed, and therefore the CO is forced to combine with Hb_2 direct—which it does much less readily, and therefore the curve falls again. An analytical expression of this fact is given below. In order to find the dissociation curve in question we have to find the relation between ξ', the CO %-saturation, and x, the O_2-tension. We know that the total amount of haemoglobin present can be represented by 100, so that, neglecting the small amounts of the unsaturated compounds present,

$$2\xi + 2\xi' + 2y = 100.$$

Putting into this equation the values of ξ and ξ' in terms of y given in equations (B) above, we find

$$2y = \frac{100}{1 + \frac{1}{2}(k_1 x + k_1' x') + kxx' + k_1 k_2 x^2 + k_1' k_2' x'^2},$$

so that, from equation (B)

$$\xi' = \frac{100\,(k_1' k_2' x'^2 + \frac{1}{2} k_1' x' + \frac{1}{2} kxx')}{1 + \frac{1}{2}(k_1 x + k_1' x') + kxx' + k_1 k_2 x^2 + k_1' k_2' x'^2}.$$

This relation between ξ', the %-saturation with CO, and x, the O_2-tension, is too complicated for simple calculation and comparison with experimental results. It can however be shown that it may possess the general qualities of the experimental curve found by Douglas and Haldane [1912, p. 279]. It rises from the horizontal axis in much the same way as their curve, with diminishing O_2-tension, and then reaches a maximum and falls again slightly as the O_2-tension becomes very small and finally vanishes. This can be simply tested by finding the value of $\partial\xi'/\partial x$ at $x = 0$, i.e. the slope of the curve at the point of zero O_2-pressure. If $\partial\xi'/\partial x$ is positive, i.e. if the curve slopes upwards at first, ξ' first increases and then of course later decreases as x increases. If $\partial\xi'/\partial x$ is negative at $x = 0$, then the curve slopes down from the beginning.

We should expect therefore, from Douglas and Haldane's results, that the value of $\partial\xi'/\partial x$ at $x = 0$ should be > 0.

A simple calculation will show that

$$\frac{1}{100}\left(\frac{\partial\xi'}{\partial x}\right)_{x=0} = \frac{\frac{1}{2}kx' + \frac{1}{2}kk_1' x'^2 + kk_1' k_2' x'^3 - k_1 k_1' k_2' x'^2 - \frac{1}{2}k_1 k_1' x' - 2kk_1' k_2' x'^3 - kk_1' x'^2}{(1 + k_1' x' + 2k_1' k_2' x'^2)^2}.$$

Let us assume that x' is very large, i.e. that there is a large tension of CO. Then $(\partial\xi'/\partial x)_{x=0}$ is obviously negative, because of the preponderating negative value of the term $-2kk_1' k_2' x'^3$. Hence Douglas and Haldane's curve will *not* exhibit a fall of CO %-saturation as the O_2-tension is diminished to zero, if the CO-tension is high. The assistance provided to the combination of CO by the presence of a little unsaturated Hb_2O_2 is of no importance if the CO-tension is very high. Let us assume that x' is very small. Then the value of $\frac{1}{100}(\partial\xi'/\partial x)_{x=0}$ is

$$\frac{\frac{1}{2}kx' - \frac{1}{2}k_1 k_1' x'}{(1 + k_1' x')^2},$$

neglecting squares or higher powers of x'. This quantity is therefore positive if $k > k_1 k_1'$. Now $k = k_1 k_3$. Hence we must have $k_3 > k_1'$. This we should expect to be the case. From the assumption already made, k_2' is very much greater than k_1', CO combines much more readily with the partially saturated Hb_2CO than with the completely unsaturated Hb_2: one

would expect it therefore to combine much more readily with the partially saturated Hb_2O_2 than with the completely unsaturated Hb_2. In that case k_3 would be as much greater than k_1', as k_2' is: and on the latter inequality the whole of the argument of this paper is based. Without therefore introducing any new hypothesis we have shown that, for a small constant CO-tension, the CO-O_2-dissociation curve in the presence of an increasing tension of O_2 rises at first, reaches a maximum and falls slowly to zero, as in Douglas and Haldane's recorded observations.

In all the above considerations Hb_2 has been taken as the general type of the molecular cluster. Any other type of molecule, Hb_3, Hb_4, ..., can be considered of course in exactly the same way : the mathematical treatment would be more complicated, although exactly the same in type.

Douglas, Haldane and Haldane in their paper [1912, p. 296] put forward an ingenious theory to account for the several dissociation curves observed, both with O_2 and CO : and they gave an equation for a dissociation curve which fits the experimental points fairly closely, but not so closely as does the equation deduced from the hypothesis of this paper. In order to arrive at this equation they made various assumptions as to the exact values of the dissociation constants of the various reactions, for which, as they admit [1912, p. 301], there are no real a priori reasons : and there are other improbabilities in their assumptions, at least as great as in those made above in this paper. For example the idea that " the aggregated molecules do not give up or take up O_2 or CO without first splitting up into simple molecules" seems difficult to admit : and it is not easy to understand, if oxyhaemoglobin molecules are continually aggregating and reduced molecules are continually aggregating, how the oxyhaemoglobin and the reduced haemoglobin molecules do not aggregate together to form unsaturated clumps. It is true however that their theory does agree to a considerable extent with observed facts, even though they have made many rather difficult assumptions in the development of it : so that, even though the theory sketched in this paper seems to me to be more probable, I do not feel that one can as yet decide definitely between the two. It is noticeable however (as Barcroft shows) that the equation to the dissociation curve given here, and in the previous paper, does definitely suit the experimental facts better than the equation deduced from the hypothesis of Douglas, Haldane and Haldane. That a modification however of their assumptions would make their equation fit the experimental facts better than at present, seems to me very probable : so that a judgment between their theory and mine can scarcely be given on the facts hitherto discussed. At present however the theory and assumptions

given here seem to me to be simpler and easier, as well as to fit the experimental data better, than the hypothesis advanced by Haldane, Douglas and Haldane.

SUMMARY AND CONCLUSIONS.

The O_2- and CO-dissociation curves of haemoglobin are known to differ, according as salts and CO_2 are present or are not. A theory to explain this has already been advanced, viz. that the simple molecules of haemoglobin are aggregated into molecular clusters. This theory explained many of the facts very exactly, but certain objections had been raised to it in particular by Douglas, Haldane and Haldane, especially in relation to the CO-O_2-dissociation curves.

It is shown here that the theory is capable of including all the known facts in relation to CO- and O_2-dissociation curves, if one is allowed to make certain simple assumptions as to the order of magnitude of the equilibrium constants in the several reactions involved. These are:

(i) that the half saturated molecules Hb_2O_2 and Hb_2CO are very unstable, and change at once into either Hb_2 or $Hb_2(O_2)_2$, $Hb_2(CO)_2$ or $Hb_2(CO)(O_2)$,

(ii) that the half saturated molecules Hb_2O_2 and Hb_2CO combine much more readily with CO than with O_2.

The first of these assumptions can be explained as due to the fact that Hb_2 is $Hb = Hb$, while Hb_2O_2 is $= Hb, Hb = O_2$, with two unsaturated bonds, which tends to combine at once with more O_2 to form $O_2 = Hb, Hb = O_2$.

If these assumptions are justified, we may deduce that since CO combines much more readily with Hb_2O_2 than with Hb_2, haemoglobin will take up more CO at a given tension, if a little O_2 is present than if O_2 is completely absent.

REFERENCES.

Barcroft (1913), *Biochem. J.* **7**. 481.
Barcroft and Hill (1910), *J. Physiol.* **39**, 411.
Douglas, Haldane, J. S. and Haldane, J. B. S. (1912), *J. Physiol.* **44**, 296.
Hill, A. V. (1910), *J. Physiol.* **40**, *Proceedings*, iv.

XLVIII. THE COMBINATIONS OF HAEMOGLOBIN WITH OXYGEN AND WITH CARBON MONOXIDE. II.

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In the preceding paper Hill [1913] has expanded his formula

$$y/100 = \frac{Kx^n}{1 + Kx^n}$$

in which y = the percentage saturation of the haemoglobin with oxygen, x the oxygen pressure, K the equilibrium constant of the reaction and n the average number of molecules of haemoglobin in each aggregate. The formula was originally put forward [Hill, 1910] to cover the dissociation curves of haemoglobin in pure aqueous solution and in certain saline solutions. With the introduction of certain assumptions it may now be applied (1) to the affinity of blood for oxygen in the absence of carbonic acid, (2) to the affinity of blood for carbon monoxide in the absence of oxygen, (3) the partition of haemoglobin between oxygen and carbon monoxide in the presence of the two gases and either with or without acids such as CO_2 in the system. The validity of the theory depends not only upon the soundness of the reasoning on which it rests, but upon the accuracy with which it fits the vast number of experimental data by which it may be tested.

It may here be pointed out that the formula contains but two constants n and k: the experimental test therefore is a much more crucial one than in the case of the more adaptable formula of Douglas, Haldane and Haldane [1912], which contains three constants.

Since the formula was first published by Hill, I have devoted a great deal of attention to the accuracy with which it fits the facts. The correspondence is so striking that I propose to record it in the present paper.

I will treat of the evidence as regards

(1) the reaction $Hb + O_2 \rightleftarrows HbO_2$,

where the haemoglobin is dissolved in solutions of various salts,

(2) $Hb + O_2 \rightleftarrows HbO_2$ with reference to blood.

HAEMOGLOBIN IN SALINE SOLUTIONS.

In Hill's preliminary communication he tabulated the comparison of the curves calculated from the formula and the freehand curves drawn by Camis and myself [1910] for solutions of haemoglobin in water, 0.7% NaCl and 0.9% KCl.

The figures lost much of their force from want of a fuller presentation. It was not evident from a casual study of these data that all the curves they represented for which the value of n was more than unity were in essence S-shaped. It therefore seemed that there was an essential difference between these curves and those which were known to exist for blood, the latter being S-shaped curves.

Blood.

Blood offers a far more searching test than haemoglobin solution because the curves are much more spread out. There is no case on record in which a great number of determinations, say more than about a dozen, have been carried out on the blood of any one person at one time. Fortunately the blood of the persons who have been most thoroughly investigated seems the same at one time as at another[1]; a single curve may be taken as representing the blood of one individual, for instance Douglas, Zuntz or myself, irrespective of the time at which the determinations were made.

Thirty-eight determinations of points on Douglas' normal dissociation curve at his existing alveolar CO_2 pressure of 40–41 mm. have been made. The series is especially valuable because the determinations have been made by somewhat different methods, by different persons, and on different scales. Rather more than half were made by me, and for all these the differential method of blood gas analysis was used, dilute ammonia unaided by saponin being employed in the blood gas bottles and in most cases 0.1 c.c. of blood being used for each analysis. Sixteen of the points shown were determined by Haldane and Douglas; they did not use the differential apparatus, but Brodie's adaptation of the old Barcroft-Haldane apparatus. They used 1 c.c. of blood for their analyses. Five determinations were made with dilute sodium carbonate as the fluid for taking the blood whilst the rest were made with ammonia. They used saponin in addition to the alkali. Moreover, Haldane and Douglas treated the gas in their tonometer somewhat differently from the way in which I did. Their method involved them in much greater corrections for the changes during analysis in gases held physically in solution. In spite of this their method was probably somewhat more accurate than mine.

[1] Barcroft [1911], Douglas, Haldane and Haldane [1912, p. 283].

In treating of the data I have adopted two methods. The first is to take all the data in order, to divide them into three groups as nearly as possible equal, to state (1) the difference between the observed and the calculated percentage saturation in the case of each observation, (2) the greatest difference in each direction and the mean difference in the case of each group and (3) the same in the case of the whole series. The following is the result:

Pressure obs.	%sat. obs.	Dev. from curve	Pressure obs.	%sat. obs.	Dev. from curve	Pressure obs.	%sat. obs.	Dev. from curve
9	6	+1	36	63	+0·5	59	84	−1
11	6	+3	36	67	+4·5	61	83	−3
17·5	26	+3	37	63	−1	61	84	−2
18	24	+1	38	65	−1	70	89	−0·5
20	27	−0·5	39	71	+3	77	92	+0·5
20	30	+2·5	39	67	−1	85	92	−1·5
23	38	+3	41	71	+1	85	94	+0·5
24	37	−1	42	75	+4	90	92	−2·5
26	39	−3	42	72	+1	95	99	+4
29	51	+2	49	77	−1·5	97	94	−1·5
30	50	−1	49	79	+0·5	96	96	+0·5
34	57	−2	53	78	−3	104	96	0
			59	81·5	−3·5	104	97	+1

Total of 38 — Greatest error +4·5 / −3·5 — Mean 0·14 %

The second plan of treating the results is to divide according to the methods by which they were obtained. Taking the 16 results obtained by Haldane and Douglas, eight are above the line and eight below it. The greatest individual discrepancies are +4% and −3%. The mean of the sixteen is 0·19%. Of their sixteen results one differs from the calculated number by 4%, three by 3%, three by 1·5–2%, eight by 0·5–1% and one by under 0·5%: whilst of the whole series of thirty-eight, there is one of 4·5%, three of 3·5–4%, nine of 2·5–3%, six of 1·5–2%, eighteen of 0·5–1% and one of under 0·5%. The general run of the figures is so nearly the same in each of the two series, as to prove that the method of experiment does not affect the comparison at issue.

Nine points have been determined on the Zuntz blood at 40 mm. CO_2 pressure. I could not find that it differed from that of Douglas, it is therefore referable to the same curve. Of these nine, three fall on the curve (within 0·5% of it), four are below it and two are above it.

Oxygen pressure	0	12	30	37	37	43	61	79	97
Observed % saturation	0	4	50	61	61·5	72	86·5	92	95
Divergence from curve	0	−5	−1	−4	−3·5	0	+·5	+1	0

Extreme divergence $\begin{cases} +1 \\ -5 \end{cases}$. Mean divergence −1·3 %.
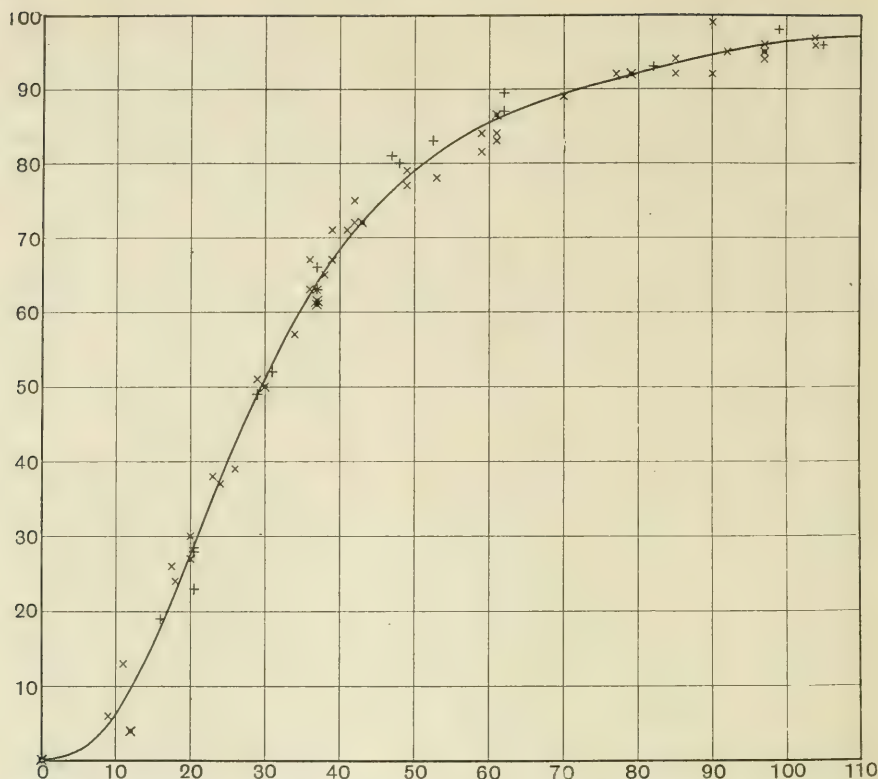
Fig. 1.	Ordinate = percentage saturation with oxygen; abscissa = oxygen pressure.
× Blood of Douglas.	✳ Blood of Zuntz.	+ Blood of Haldane.

Referable to this curve also is the blood of Haldane which I have taken from the figure in the *Journal of Physiology*, vol. XLIV, page 283, as faithfully as the scale of the figure permits. The points are as follows:

Oxygen pressure	26	20·3	20·5	20·5	29	31	37	37	47	48	52·5	62	62	82	99	105
Observed % sat.	19	28·5	28	23	49	52	63	66	81	80	83	87	89·5	93	98	96
Div. from curve	0	−0·5	0	−5	0	+0·5	−1	+2	+4	+2	+1	0	+2·5	+1	+2·5	0

Extreme divergence $\begin{cases} +4 \\ -5 \end{cases}$.	Mean divergence 0·6 %.

The results of the comparisons between the curve drawn from the equation, and the actual determinations which I have cited may be summarised as follows. (1) Of the 63 determinations none differs by more than 5 % from the calculated value, (2) the "average error" reckoning all departures from the calculated value as positive quantities is 1·6 %, (3) the mean of all the errors reckoning those above the line as positive and those below it as negative is 0·02 %.

It is known that the blood of different persons differs. I have compared the points which have been determined on my own blood with the curve, the constants of which are $n = 2\cdot5$, $K = 0\cdot000292$. Only about 20 points are available for the comparison.

Dividing them into two groups they are as follows:

Pressure of oxygen	0	1·5	7	13	22	28	36	36	37	38·5
% sat. observed	−1	2	4	12	37	55	69·5	71	68	72·5
Div. from calcd. value	−1	+1·5	0	−4	−2	0	0	+1·5	−2·5	+0·5

Extreme divergence $\begin{cases} +1\cdot5 \\ -4 \end{cases}$.   Mean $-0\cdot6\,\%$.

Pressure of oxygen	38·5	43	50	50	50	50	57	65	65	65	89
% sat. observed	74·5	79·5	83·5	83·5	84·5	85	89	89	90	90·5	95
Div. from calcd. value	+2·5	+1	−0·5	−0·5	+0·5	+1	0	−3	−2	−1·5	0

Extreme divergence $\begin{cases} +2\cdot5 \\ -3 \end{cases}$.   Mean $-0\cdot23\,\%$.

Taking the two series together the results may be summarised as follows: (1) no single determination differs by more than $4\,\%$ from the calculated value, (2) the "average error," calculating all errors as positive, is $1\cdot2\,\%$, (3) the mean divergence, calculating points above the line as positive and those below as negative, is $0\cdot36\,\%$.
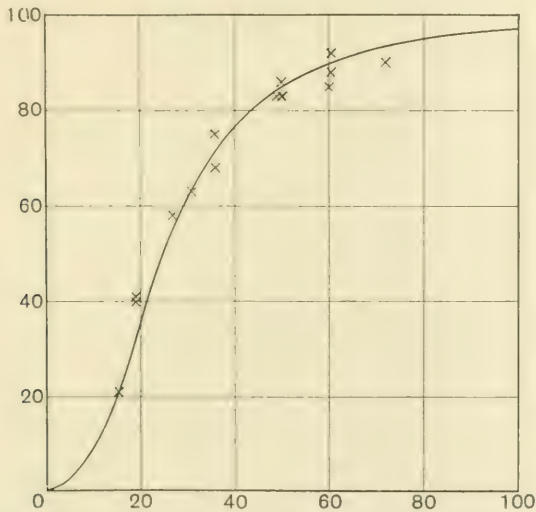
Fig. 2. Zuntz[1] dissociation curve, 35 mm. CO_2; ordinate = percentage saturation with oxygen, abscissa = oxygen pressure in mm.

Of the cases of human blood which have been studied at all exhaustively by me, the least satisfactory from the present point of view is that of Zuntz at his existing alveolar CO_2 pressure of 34—35 mm. of CO_2; here, however,

[1] The data and pressure given on p. 62, *Journal of Physiol.* Vol. XLII, are slightly erroneous owing to a wrong correction for the zero of the gas burette.

the chief cause of discrepancy is a point done in duplicate (pressure 19 mm. percentage saturation 41 and 40 %). The data are as follows. The curve here is very steep, and a slight error in the pressure measurement would account for it.

Pressure of oxygen	15·5	19	19	23	27	31	36	36	49	50	50	60	60·5	60·5	72
%/o sat. observed	21	40	40	50	58	63	75	68	83	86	83	85	92	88	90
Div. from calcd. value	−1	+6	+7	+4	+3	0	+4	−3	−1	+1	−2	−5	+2	−2	−3

Of these fifteen points one is on the line, seven above and seven below it, the average error counting all errors as positive is 3 %, whilst the mean counting errors above the line, as positive, and those below it, as negative, is 0·66 %.

So far as I know there are no other cases in which a sufficient number of points has been determined and these spread sufficiently uniformly over the curve to test the truth or otherwise of the theory.

At this point a few words may be said by way of comparison between the curve obtained from Hill's formula and that obtained from the formula of Douglas, Haldane and Haldane. Over a great portion of the curves obtained from the two formulae they agree so closely as to make an experimental test between the one and the other almost impossible.

At pressures below 20 mm. the two curves differ and it may be well to compare this portion of them with the observed points. This task is made easier because at very low oxygen pressures the difference between the blood of different persons is very slight and therefore the determinations—all too few—for human blood may be taken together for the purpose. This may be done the more freely by me because such error as there is in the application of results obtained from my blood to the curves of Douglas' blood which are those depicted, would favour the formula of Douglas, Haldane and Haldane.

Below 15 mm. there appear to be eleven determinations on human blood, of these the three lowest, at 0 and 1·5 mm. offer no test of the relative merits of the two curves, yet I have included them because they are all important to the argument as showing that the percentage saturations as observed start from the zero. Had there been some such error as incomplete laking of the blood or wrong correction for the gases in physical solution these points would not have fallen on the base line.

Below are the data of the eleven points.

Pressure of oxygen	0	0	1·5	6	7	8	9	11	12	12	13
Percentage saturation observed	0	−1	2	−1	4	+1	6	13	4	10	12
Divergence from Haldane's curve	0	−1	+1	−6	−3	−7	−3·5	+1	−10	−4	−3·5
Divergence from Hill's curve ...	0	−1	+2	−2·5	+1	−2·5	+1·5	+5·5	−5	+1	+1

Averaging the points between 5 and 15 mm., (1) the extreme departures from Haldane's formula are $+1$ and -10 and from Hill's $+5·5$ and -5, (2) reckoning the "average error" (counting all the errors as positive) it is with regard to Haldane's formula $4·8 \%$ and with regard to Hill's $2·5 \%$, (3) reckoning errors above the line as positive and below it as negative, the mean divergence from Haldane's curve would be $4·5 \%$ and from Hill's curve $1·2 \%$. It seems clear from the figures and more clear from Fig. 3, that Hill's formula more closely represents the facts than does that of Haldane. Eight points is however a small number to take for such a purpose and while it is evident that the equation $y/100 = \dfrac{Kx^n}{1 + Kx^n}$ has the better of the comparison, many more points would have to be determined before the argument could be regarded as settled on this count.
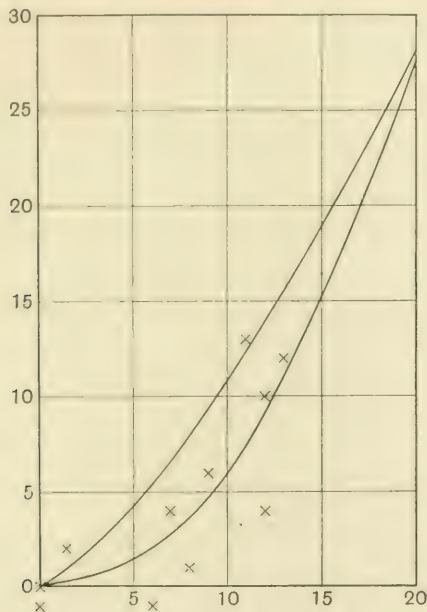
Fig. 3. Lower portion of dissociation curves of human blood. The upper curve is drawn from Douglas, Haldane and Haldane's formula, the lower curve from Hill's formula. Ordinate = percentage saturation with oxygen; abscissa = oxygen pressure in mm.

It may be taken as proved that either by accident or otherwise the curves for human blood at normal alveolar CO_2 pressure closely follow Hill's equation, which is founded upon a physical conception. The natural test is to see whether curves obtained under widely different conditions also agree with the formula.

The simplest way of varying the curves is to alter the concentration of the carbonic acid. In this way a whole series of curves may be produced as was shown by Bohr, Hasselbalch and Krogh [1904]. Poulton and I [1913] investigated the question of whether three curves of the series other than the normal curve at 40 mm. pressure could be represented by Hill's formula. The answer was striking for not only were the curves capable of expression by the general formula $y/100 = \dfrac{Kx^n}{1 + Kx^n}$, but over the whole series only one of the two constants (K) varied, n remaining at a constant value throughout. Hence it is possible from a graph relating the concentration of carbonic acid to the value of K to obtain the dissociation curves for concentrations of carbonic acid intermediate between those at which the determinations were actually made. As this has proved useful on many occasions I append the diagram.
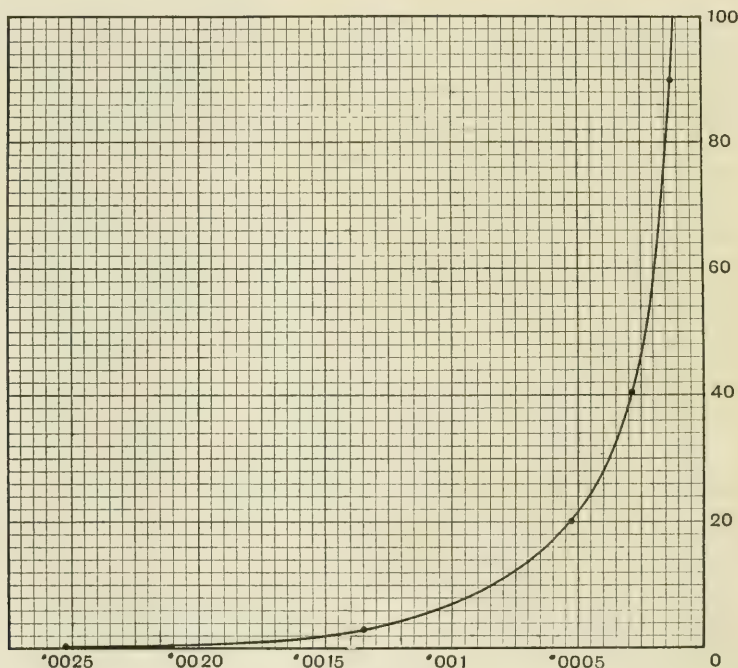
Fig. 4. Ordinate = CO_2 pressure in mm. Abscissa = K.

It was shown by Orbeli and me [1911] that carbonic acid had no specific effect in changing the affinity of haemoglobin for oxygen, but that it shared this property with lactic acid: Mathison subsequently showed that other organic and inorganic acids had a like effect.

The following points were obtained by the addition of lactic acid (0·2 °/₀) to blood from which the carbonic acid had been as completely as possible removed by shaking.

Pressure of oxygen			10	20	20	30	30	40	50	50
Percentage saturation observed		...	11	39	40	64	68	82	87	86
Divergence from calculated results			+1	−1	0	0	+4	+2	−1	0

$n = 2·5, \ K = 0·000363$
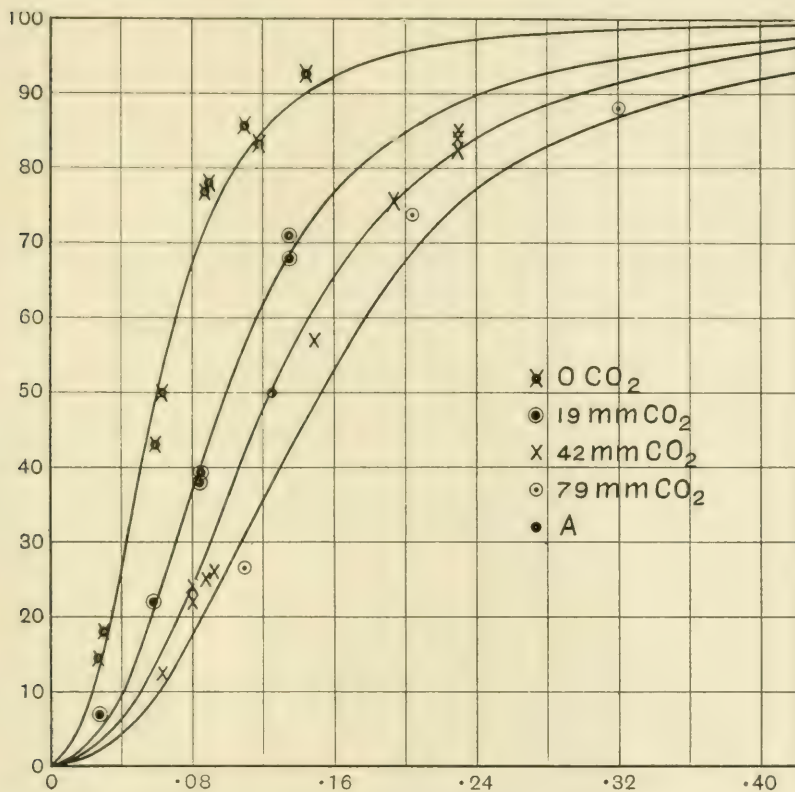
Fig. 5. Equilibrium curves of CO haemoglobin drawn from Hill's formula. The points are the observations of Douglas, Haldane and Haldane. Ordinate = percentage of haemoglobin with CO; abscissa = CO pressure in mm.

In addition to this, curves might be cited in which both lactic and carbonic acid were added to blood, such curves for example as those given by me in the record of my work in Teneriffe and by Orbeli and myself on the effects of low oxygen pressures on animals. The points on curves all follow the equation with the same degree of exactitude as those cited in this paper, but the points determined in each case were too few to make any detailed discussion of them worth while.

Most of what has already been said as regards oxyhaemoglobin curves might be repeated with regard to CO-haemoglobin. Fortunately a very complete series of data on this subject has recently been published by Douglas, Haldane and Haldane. In Fig. 5, these data are shown and along with them a system of curves drawn from Hill's formula. The scale on which the diagram is plotted is such that the centre point marked A of the curve corresponding to 40 mm. CO_2 would be on the same place in the figure as the centre point of the oxyhaemoglobin curve corresponding to the same CO_2 pressure. That being so it will be found that the whole figure is superposable upon the same figure for oxyhaemoglobin. Not only are the points then faithful to Hill's formula, but on a different scale the relation of K to the CO_2 pressure is the same in the case of oxy- as of CO-haemoglobin.

There is one more point to which I should draw attention, and which is involved in the fidelity of the observed points to the theoretical lines. Did the lines in Fig. 5 actually conform to Haldane's formula, there would have been the same slight discrepancy between the lines and the points at the bottom of the diagram in the case of CO-haemoglobin as in the case of oxy-haemoglobin and to just the same extent. When this difference reappears in another series such as the CO-series in which the points have been arrived at by wholly different methods (these were obtained by the method of carmine titration) the weight of evidence in favour of their having a real significance is greatly increased.

CONCLUSION.

1. The available data for the dissociation curves of blood agree very closely with the theoretical curves deduced from the following physical conceptions:

(a) That the reaction between haemoglobin and oxygen is a reversible chemical change $Hb_n + nO_2 \rightleftarrows Hb_nO_{2n}$.

(b) That n is the average number of molecules aggregated together, the value of n depending upon the nature and concentration of the electrolytes in the solution.

(c) That the effect of acids is to change the equilibrium constant of the reaction without sensibly altering the degree of aggregation of the molecules.

(d) That the above reaction does not involve the breakdown or reformation of the aggregates.

(e) That unsaturated oxides are unstable and break up into haemoglobin and saturated oxides.

2. The available data with regard to the reaction of CO and oxygen support an entirely similar conception of carboxyhaemoglobin.

3. So far as the curves deduced from the formula $y/100 = \dfrac{Kx^n}{1 + Kx^n}$ (Hill) can be distinguished from those yielded by the formula

$$x = \frac{K_y \lfloor 1 + b\,(1 - y)\rfloor}{(1 - y)\,(1 + ay)} \quad \text{(Douglas, Haldane and Haldane)},$$

the experimental evidence leans towards the former.

REFERENCES.

Barcroft (1911), *J. Physiol.* **42**, 47.
—— and Camis (1910), *J. Physiol.* **39**, 118.
—— and Orbeli (1911), *J. Physiol.* **41**, 355.
—— and Poulton (1913), *J. Physiol.* **46**, *Proceedings* vi.
Bohr, Hasselbalch and Krogh (1904), *Skand. arch. Physiol.* **16**, 402.
Douglas, C. J., Haldane, J. S. and Haldane, J. B. (1912), *J. Physiol.* **44**, 275.
Hill, A. V. (1910), *J. Physiol.* **40**, *Proceedings* iv.
—— (1913), *Biochem. J.* **7**, 471.
Mathison (1911), *J. Physiol.* **43**, 347.

XLIX. SEPARATION OF PROTEINS.
PART III. GLOBULINS[1].

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Up till the middle of last century it was generally held that the protein of blood serum and other kindred fluids was a single homogeneous substance —albumin. The word globulin, apparently first used by Berzelius, was applied by him to two substances, the protein part of haemoglobin (called by him haemato-globulin) and the protein of the lens, and came thus to be associated with cell-protein. That the serum protein could be split up, or that serum contained more than one protein was first suspected on account of some experiments of Liebig, Zimmerman and others, who found that great dilution of blood-serum produced a precipitate, especially after neutralisation with acetic acid. It was, however, Panum [1851] of Copenhagen who first discovered serum-globulin, and who developed the now classical method of preparation by first diluting the serum with water, and then adding acetic acid. Panum showed that the substance occurs constantly in human blood both in health and in disease, and called it serum casein from its resemblance to milk casein. Shortly after Zimmerman [1854] published the carbonic acid gas method of preparation. A few years later Alexander Schmidt [1862] in the course of his extensive observations on serum and kindred fluids showed in all cases that, side by side with the more soluble albumin, there was always the more insoluble globulin. In pursuance of his views on blood clotting he called the globulin in serum fibrino-plastic substance, though as often as not in his writings he calls it globulin.

Considerable activity followed on the publication of Schmidt's views: several ways of producing protein precipitates in serum were discovered and discussed; and there was some little confusion as to whether there was more than one substance, and as to nomenclature, till Heynsius [1869, 1876] gave

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[1] Part I Haslam [1905], Part II Haslam, [1907].

good reasons for believing that, whether precipitated by simple dilution, carbonic acid gas, dilute acids, or saturation with sodium chloride there was only one substance—para-globulin as it was then called—a conclusion that, except in regard to sodium chloride, still holds good.

In 1878 Hammarsten [1878] published his great attempt at the purification and estimation of this substance. He introduced magnesium sulphate which produced a much larger precipitate than the reagents hitherto employed. In 1883 Burckhardt [1883], in repeating Hammarsten's experiment showed that, in addition to the globulin which was insoluble in water, a water-soluble substance was contained in the magnesium sulphate precipitate. Thus the probability of the existence of a third protein in serum was shown, though Hammarsten maintained that the magnesium sulphate precipitate was a single substance. Burckhardt's observation was, however, confirmed by Marcus [1899].

Meanwhile Hofmeister had conceived the idea of dividing serum and other liquids into fractions by means of taking precipitates at different degrees of concentration of one and the same salt solution, and thus isolating the different proteins. The first attempt was carried out by Kander [1886] when he precipitated globulin by half-saturation with ammonium sulphate, and albumin by complete saturation of the filtrate, the globulin roughly corresponding to Hammarsten's. Later on this system was elaborated by Pick [1902], Fuld and Spiro [1900], and others, both in the case of albumoses and serum. It was at first found that the globulin brought down at half-saturation could be split into two portions. The first was found to resemble the original water-insoluble substance of Panum, Schmidt, Heynsius and others, and was accordingly named eu-globulin; the second was more soluble and was called pseudo-globulin. In a further research Porges and Spiro [1903] thought there were three distinct fractions, both by ammonium, sodium, and magnesium sulphate: and Reiss [1904] also in Hofmeister's laboratory, decided on three—eu-globulin, pseudo-globulins I and II. Freund and Joachim [1902] on the other hand, taking the two fractions eu- and pseudo-globulin, showed that each contained a water-soluble portion, and took the view that there were four globulins. In regard to this system of fractionation I showed by direct experiment in Part I [1905] of this series that no single precipitation, both in the case of albumoses and serum proteins, whether by acid, salts of heavy metals, salting out or alcohol, ever produced a complete separation: that in the precipitate the substance of the filtrate could always be demonstrated, often up to 20 or 30 %; and similarly, mutatis mutandis, with the filtrate. And Wiener [1911], working on quantitative lines, showed that

reliable estimations of globulin could not be made by single precipitations. J. Mellanby [1907] from a determination of the percentage of protein at gradually increasing concentration of alcohol drew the conclusion that there were three different proteins in serum. Fractional methods, then, seemed to have left the subject in a more confused state than that in which they found it. I believe, however, and hope to show in these pages, that the method is inherently sound, and must for the present be regarded as one of the most important, and in many cases the only, method, we have of separating proteins.

Fractional precipitation.

The fundamental observation on which the method rests is that when a precipitant is added gradually to a protein fluid, and the resulting precipitate collected in successive portions, it is found that one portion differs from another. It is inferred from this that a separating process has been set up. And the proof of the correctness of this inference is found in the fact that if the process is continued sufficiently, substances that are undoubtedly distinct from each other can be obtained. Now in the case of the precipitant being a salt such as ammonium sulphate each increment of the salt in the protein fluid is followed by an increment of the precipitate. It is possible that at some concentrations the precipitate falls rather more thickly than at others: and indications may thus be afforded as to how a separation may be attempted. But it is entirely fallacious, as I have already pointed out, to suppose that one protein is wholly, or even nearly wholly precipitated before the next begins to come down. If there are two or more proteins their precipitation commences almost if not quite simultaneously, and they continue to come down together till the end of the precipitation, though at any one time different quantities of each might be coming down. Mellanby has demonstrated this point in the case of serum and ammonium and magnesium sulphate, having made quantitative experiments and plotted curves therefrom. He finds the precipitate falls very uniformly and that at no point is there a cessation of precipitation. His conclusion, however, that no splitting up of the serum can be brought about by these means, is erroneous. If no separation were brought about by the salting out, the protein in any one fraction would resemble that in every other. But this is obviously not the case. The chief cause of confusion hitherto has been of the opposite kind; that there has been no way of determining in the case of differing fractions, whether each fraction connotes a separate substance or not.

Let us suppose that there are two substance A and B; that A in a pure

state is precipitable more easily and is all precipitated at half[1] saturation ; while B, in a pure state, does not commence to come down till the half-saturation point is reached, and only comes down completely on full saturation. Suppose now that three successive precipitates are taken at one-third, two-thirds, and full saturation. The portions would be composed as follows :

Fraction 1.	n_1 parts A,	m_3 parts B.
2.	n_2 parts A,	m_2 parts B.
3.	n_3 parts A,	m_1 parts B.

where $n_1 > n_2 > n_3$ and $m_1 > m_2 > m_3$.

Each fraction would thus show some difference from every other. Let us suppose that each fraction is redissolved and again precipitated at the same concentration. The three fractions would then differ from each other more markedly. The first would contain a greater proportion of A ; the last would contain a greater proportion of B ; while the middle one would remain about the same, much the greater part coming down between the precipitation limits. Generally speaking the separating power of the salt is not sufficient to cause any great change at any one precipitation. Thus far we might consider that we were dealing with three separate substances.

There are two principle methods by which to determine whether a fraction represents a substance or not.

1. Constancy of quantity under repetitions of the process. Fractions 1 and 3 would show this constancy after a time. Fraction 2 consisting of a mixture, would not, but would gradually disappear.

2. Subdivision of a fraction to find whether it is consistent or not. Fraction 2 on subdivision would show that it consisted of substances properly belonging to fractions 1 and 3. At half-saturation, in short, it can always be divided into a precipitate containing a higher proportion of A than B, and a filtrate containing more B than A.

Having decided on the number of substances into which the parent body can be split, there next arises the question as to how far each can be separated from the other. In the case of A, after a certain number of precipitations, the filtrates contain a constant quantity of organic nitrogen : that is to say, the separating process has entirely ceased. The separation, therefore, is presumably complete, but I will return to this point later. In regard to B, when we arrive at the point that half-saturation produces no precipitate, we cannot infer that the separation is complete. I have shown in previous

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[1] As in previous papers I follow the usual convention of describing as "half" saturated a solution made by mixing equal volumes of saturated salt and protein solution. In reality such a solution is less than half saturated.

papers that, if a further small addition of salt be made, the resulting precipitate will consist largely of A ; that is to say, a quantity of A remains dissolved in the B fraction. Means must be adopted, therefore, to continue the separation. Hitherto, the procedure has been to take a small fraction, dissolve it, and reprecipitate at the same concentration. The precipitate consisting mostly of A, is withdrawn, while the filtrate is returned to the main solution. This may be continued until no further trace of A can be found in the fractions. The separating process, then, comes to an end in this case also. But although the separating process has come to an end we cannot assume that the substances are completely separated, because in analogous separations in fractional distillation, crystallisation and precipitation it may happen that a certain amount of the substance being got rid of remains with the substance being purified, the two together, in this instance, acting as one substance towards the separating agent. Proof positive, therefore, can only be obtained by means of independent reactions. In the case of most proteins these, at present, are few in number: it is obvious that some separation must precede the discovery of typical reactions based on constitutional differences. In one of the separations to be described, we have such a reaction, and it goes far, I think, in demonstrating the validity of fractional precipitation in the case of serum proteins.

In regard to the question of the chemical individuality of the products of such separation, it can only be said that it is convenient to regard them as individuals until they are shown to be capable of further subdivision. Each can be tested with all the means at our command.

First Separation.

In the case of serum (ox-serum was used in all these experiments) my first procedure was to divide it by means of half-saturation with ammonium sulphate. The serum was diluted some four times with water, and an equal volume of the saturated salt solution was added. The resulting precipitate was collected, redissolved in water, and the process repeated until the separation, as shown by the sulphuric acid decomposition test[1], was complete. The separation was an easy one at this concentration ; three or four precipitations got rid of all but a small quantity of albumin, and the

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[1] 8–10 c.c. of the filtrate are mixed with an equal volume of concentrated sulphuric acid in a test-tube and warmed to boiling point. As the protein in the filtrate diminishes the tint gets lighter and when two successive filtrates give the same tint the amount of protein in the filtrates has become constant and the separation is at an end.

separation was completed in some six precipitations. The globulin fraction so obtained was almost entirely soluble in salt and water.

I then sought to divide it further by ammonium sulphate. Precipitation at one-third saturation (one volume of saturated solution being added to two volumes of the globulin solution) was carried out, and in this way the protein was divided into two fairly equal portions, the greater portion, perhaps, being that precipitated. The precipitate was re-dissolved and re-precipitated; and the process was repeated until the filtrate showed only small quantities of protein. During the progress of the precipitations, a larger and larger portion of the precipitate became insoluble in salt and water, and the experiment, on this account was not continued to a point of constancy in the usual way, since the insoluble matter might have held to itself soluble protein and so have vitiated the result. It served to indicate, however, that the globulin of half-saturation could be further divided into a water-insoluble part, precipitable at one-third saturation, and a more soluble portion not so precipitable. The latter was then treated by the process of fractional precipitation to remove any water-insoluble globulin that had remained dissolved in it. Powdered ammonium sulphate was added gradually and dissolved by stirring until a small precipitate, say some 15–20 % of the amount of protein present appeared. This was filtered off, redissolved in water, and saturated salt solution was added till the solution was at "one-third" saturation. This caused precipitation of a good proportion of the fraction. Examination showed it to consist largely of water-insoluble globulin. The filrate from the precipitation was returned to the main liquid, and the whole process was repeated some five times, the last fraction showing only quite a small amount of globulin. A further small amount of globulin was removed by dialysis.

The resulting substance was easily and completely soluble in water, and could be precipitated at half-saturation with ammonium sulphate. It was tested for albumin. A portion was dissolved in 50 c.c. water and precipitated by the addition of 50 c.c. saturated salt solution. This precipitate was redissolved and re-precipitated in the same way at the same volume[1].

> 90 c.c. of 1st filtrate gave by Kjeldahl 1·0 c.c. $N/10$ NH_3,
> 90 c.c. ,, 2nd ,, ,, ,, 0·95 c.c. ,, ,,

The small amount of protein in the filtrate, being constant in quantity, is presumably pseudo-globulin.

It was mostly precipitable by saturation with sodium chloride and nearly completely by magnesium sulphate. With ammonium sulphate most of it

[1] For details of this method see Haslam [1905].

(say five-sixths) was precipitated by the addition of only three-quarters of a volume of saturated solution (43 % saturation). Half-saturation precipitated all but traces. This, then, was the pseudo-globulin of Hofmeister, the existence of which was indicated by the experiments of Burckhardt and Marcus. Its solubilities are intermediate between those of the water-insoluble globulin and albumin.

I next proceeded to ascertain whether this substance could be split up further by ammonium sulphate. Freund and Joachim found that the pseudo-globulin of Hofmeister was partly soluble, partly insoluble in water : but the insoluble portion was probably merely globulin left dissolved, as we have seen, in the pseudo-globulin. It was possible, however, that it could be split into two soluble bodies after Spiro and Reiss. A portion was divided into two roughly equal parts by ammonium sulphate in the following way. A dilute solution (about 0·25 %) was made. A small test portion was completely saturated with salt and placed in a cylindrical beaker. Saturated salt solution was then added to the main liquid until a small amount, double the volume of the test portion, placed in an exactly similar cylindrical beaker, showed on looking downwards the same degree of density of precipitate. I then assumed that about half the protein was precipitated. This, after being allowed to stand, was filtered off and the filtrate saturated to obtain the remainder. These two fractions closely resembled each other in their solubilities. Each was largely precipitable on the addition of three-quarters its volume of saturated salt solution, and the portions left over in solution appeared in no way different from those precipitated. Nor did further treatment on fractional lines show that the substance could be divided.

Since we had a water-soluble globulin commencing to fall at 33 % saturation, and nearly finishing at 43 %, that is to say when free or nearly free from other proteins, it might be supposed that a substance existed that commenced to fall at 43 % and was mostly precipitated at 50 % saturation. It might be supposed that if such a substance existed it might be nearly or entirely lost in the series of precipitations at 50 % in which albumin is got rid of. To test this point, I began afresh with some serum and endeavoured by suitable fractionations to find such a body ; but entirely without success.

Experiment. 300 c.c. ox-serum were taken and diluted to 1000 c.c. and 500 c.c. saturated salt solution were added to get rid of some of the globulin. To the filtrate were added some 700 c.c. (over half-saturation) further of saturated salt solution, so that most of the searched for substance should be precipitated while as much albumin as possible should be left in the filtrate. The precipitate was dissolved again in 1000 c.c. water and the above

precipitations were repeated. The protein resulting was then dissolved in 900 c.c. water and 500 c.c. of saturated salt solution were added so that not only globulin but pseudo-globulin might be largely precipitated also. To the filtrate was added salt solution again to make rather more than half-saturation. I proceeded in this way to eliminate gradually portions of globulin and pseudo-globulin, on the one side, and albumin, on the other; always being careful to work well outside the precipitation limits of the body for which I was looking, so that as little as possible might be lost. No such substance, however, could be found. The process was continued until a quantity too small to work with remained, and up to the last, pseudo-globulin on the one hand and albumin on the other could be demonstrated. This experiment was repeated on another sample of ox-serum.

I concluded, therefore, that no substance exists in ox-serum precipitable between 43 and 50 % salt concentration, at any rate in quantities comparable to globulin and albumin.

Water-insoluble globulin.

As it was not possible to carry to completion the separation of globulin by means of ammonium sulphate, owing to its becoming increasingly insoluble in the salt solution, I tried precipitations with acetic acid. Some water-insoluble globulin that had been prepared from ox-serum by a few precipitations at one-third saturation and then diluting with water, was shaken well with water, thrown on the filter, and washed with water. It was then suspended in water, and a few drops of ammonia were added. The globulin completely dissolved. The volume of the solution was 400 c.c. About 0·65 g. of globulin was recovered at the end of the experiment, so that if we allow for some 20 % of other proteins and loss during the experiment, there would be some 0·8 g. protein. Thus the strength of the solution was about 0·2 %.

The globulin was then precipitated by the addition of dilute acetic acid. The precipitation began when the solution was about neutral, and was rapidly completed on the addition of further acid, the solution being finally faintly acid. The mixture was allowed to stand over-night. The precipitate was then filtered off, suspended in water, and redissolved by the addition of ammonia. Normal solutions of ammonia and acetic acid were used, the quantities required being added out of burettes. Before precipitation the volume of the solution was always made to 400 c.c. 16 c.c. alkali were used to dissolve: the precipitate was usually complete on the addition of some

13 to 14 c.c. of acid, but 16 were added.   On one occasion a small excess was added, but no re-solution of the precipitate occurred.   It was noted that the globulin took more time to dissolve as the experiment advanced.

In the second filtrate the presence of water-soluble protein was easily demonstrated.   The fourth and fifth filtrate each showed a faint cloud on boiling, and a faint precipitate on saturation with salt.   In each, however, by saturating the whole with salt, and collecting the small quantity of protein obtained, water-soluble protein could be demonstrated.   The eighth and tenth filtrates were examined quantitatively for organic nitrogen by the method described in previous papers.

Eighth filtrate volume 345 c.c. gave 2·4 mg. N.
Tenth   ,,   ,,   343 c.c. ,,   2·4   ,,

Thus in precipitating the water-insoluble globulin by acetic acid a point can be arrived at in which the organic nitrogen in the filtrate is constant and the soluble pseudo-globulin presumably completely eliminated.   Reckoning that globulin contains $16\,^{0}/_{0}$ N, the amount in these filtrates would be 0·0073 g. or $0·004\,^{0}/_{0}$.   It may be noted that the separation of globulin from pseudo-globulin is considerably more difficult than the separation of pseudo-globulin from albumin.

Physical Changes caused by Separation.

The substance thus obtained is distinctly less soluble than that prepared more rapidly by a smaller number of precipitations.   Some of the essential peculiarities of globulin are, however, preserved.   The addition of alkali causes it to swell up into a jelly-like consistence.   It is, on the other hand, only slightly soluble in salt solutions.   But the question of the alteration of the physical properties of proteins on precipitation is a difficult one.

Globulin prepared by a single precipitation with acetic acid from diluted serum is a comparatively soluble substance.   It also has mixed with it considerable quantities of pseudo-globulin and albumin.   The more it is precipitated, the more insoluble does it become, and it is very generally believed that this is due to a physical alteration.   But it is not necessary to postulate physical alteration, at any rate, to the degree generally done.   Globulin can be held in solution by the other proteins of serum.   This can be shown by taking a portion of serum-protein, by precipitating with ammonium sulphate at half-saturation twice or thrice and dialysing until all the salt is removed.   A certain amount of globulin will be precipitated; but no matter how long the clear solution from which the precipitate may be

removed is allowed to dialyse, water-insoluble protein in large quantity can be demonstrated therein. Or, more simply, serum may be dialysed indefinitely and after filtering off the resulting precipitate, large quantities of globulin can be found in the filtrate. Again, during the course of fractional experiments, globulin is commonly found in solutions half-saturated and more than half-saturated with ammonium sulphate, there being excess of other protein present. Indeed, it is this property that makes the whole difficulty of the separation. It is to get rid of the globulin that remains dissolved in the pseudo-globulin that the many precipitations at high salt concentration have to be resorted to. It is this property that makes it impossible by any one precipitation by any agent to obtain more than a proportion of the globulin present. Pseudo-globulin and albumin are, in fact, the chief solvents of globulin in serum. But in this case it is clear that in any series of precipitations for its preparation and purification, where it loses at each precipitation a portion of the other protein, it loses a solvent. The more the other proteins are withdrawn from it, the more insoluble must the preparation become. Quite apart, therefore, from any changes that may go on in the globulin as a result of the physical action of the reagent used, we see that any preparation must become more insoluble as it becomes purer. But there is little doubt that some of the various reagents used may also cause insolubility. This is evident from the different behaviour of globulin when precipitated by acetic acid or ammonium sulphate. With ammonium sulphate, as we have seen, globulin gradually becomes so insoluble as to resemble coagulated protein. It may be noted that this change does not occur uniformly throughout the globulin, but a small portion of such insoluble matter appears, and continues to increase as the precipitations proceed. We may therefore argue that, apart from its separating power, ammonium sulphate has an action on globulin tending to render it insoluble. With acetic acid such action is extremely small; after many precipitations only quite a small proportion of this very insoluble matter appears.

But although it is quite easy to prepare solutions in which globulin is held in solution by the other proteins, it is apparently difficult to reproduce this condition when the various proteins have been separated from each other. At any rate, it cannot be produced by the simple addition of globulin to a solution of albumin or pseudo-globulin. I have no quantitative experiments on this point, but it is quite clear that only small quantities, if any, of completely separated globulin dissolve directly in watery solutions of separated albumin or pseudo-globulin. So far as my experiments have gone, it seems to be the case that, after a small degree of separation, say two or

three precipitations, the component parts can be restored to the *status quo* ; but that the farther the separation is pushed, the more difficult does the restoration become. This would seem to show that each act of separation causes some secondary molecular change in the parts separated.

In this connection I may recall the remarkable fact to which Hardy draws attention : that while serum itself can be readily filtered through a porous pot, globulin, even in the early stages of preparation, cannot. Hardy also takes occasion to point out that neither alkali nor salt is capable of producing so high a grade of solution as that of globulin as it exists in serum, and that a further dissolving agent must be present. To say that that agent is the other serum protein is not very different from saying that there is some sort of combination among them ; a conclusion to which Hardy is led on other grounds. In the first paper of this series I brought forward some evidence that this seems also to be the case with the albumoses.

Allowing, however, for the greatest amount of change in the process of separation, it can hardly be supposed that any radical change of constitution occurs: the changes are extra- rather than intra-molecular. So that from the point of view of chemical analysis, the matter is not of great importance.

Possibility of Substances between Globulin and Pseudo-Globulin.

In the series of precipitations at one-third saturation, it was found that, even after seven or eight precipitations at sufficient dilution, the product obtained was largely soluble in water. After dialysis a good proportion, perhaps 50 %, remained in solution. The clear solution certainly contained some pseudo-globulin, and might well have consisted entirely of this and globulin. We have seen that pseudo-globulin cannot be further split up, but there was a possibility that the bulk of the soluble matter consisted of a third substance, more soluble than globulin, but precipitable at one-third precipitation with the globulin. To ascertain whether such a substance existed, experiments were conducted on the following lines. Diluted serum is precipitated some eight or nine times at one-third saturation : this gets rid of all albumin, and a considerable quantity of pseudo-globulin. Globulin is then removed by fractional precipitation, care being taken to remove only insoluble matter, all doubtful portions being returned to the main solution, so that the quantity of substance sought for should not be diminished in this way.

This leaves us with a more soluble substance again, and from this, more pseudo-globulin is removed by precipitation at one-third saturation.

Thus alternately portions of globulin and pseudo-globulin are removed

from the protein under examination. This can be continued until the substance sought for is found, or there is no more substance to work with.

Two separate experiments were carried out, and on several occasions portions of protein were obtained which were precipitable at "one-third" saturation, and soluble in water—further examination, however, always showed them to consist of globulin and pseudo-globulin. I select the following for quotation.

500 c.c. ox-serum were diluted to 2,000 c.c., and a precipitate formed by the addition of 2,000 c.c. saturated salt solution. The precipitate was dissolved in 2,000 c.c. and a further precipitate formed by the addition this time of 1,000 c.c. saturated salt solution (one-third saturation). This latter precipitation was repeated eight times when the filtrates were found to contain only quite small quantities of protein. During the series of precipitations large quantities of insoluble matter appeared, and were removed from time to time. The final precipitate contained a fair proportion of protein that was soluble in water. From this I sought to remove any water-insoluble globulin that it might contain by means of the system of fractional precipitation before described. The protein was dissolved in 200 c.c. water (the first series of precipitations had considerably reduced it in bulk), and some 60 c.c. saturated solution were added. The small precipitate that fell was found to consist largely of water-insoluble globulin, but mixed with it was a certain amount of water-soluble substance, and this was returned to the main solution. To this a further quantity of salt was added and another fraction obtained, which was treated in the same way. Some six fractions were taken and in the last two only quite small quantities of water-insoluble matter were found. The experiment was accordingly brought to an end by nearly complete saturation of the liquid, and the resulting protein was examined. On being dissolved in 80 c.c. of water it was found that on addition of 40 c.c. saturated salt solution (one-third saturation), only some two-thirds of it were precipitated. The remainder came down on half-saturation. It was clear, therefore, that pseudo-globulin was present to a considerable extent. A fresh series of precipitations at one-third saturation was therefore undertaken, some eight in number. The resulting substance of which there was only a small quantity, was again found to contain both water-insoluble and water-soluble parts. Owing to the withdrawal of a large quantity of pseudo-globulin, the insoluble globulin was easily demonstrated again. The water-soluble part, after further precipitations of a similar character to those already described, was shown to contain both water-insoluble globulin and pseudo-globulin.

33—2

It was thus seen that no body precipitable at one-third saturation and at the same time water-soluble, could be obtained that remained constant in quantity, and could not be resolved into globulin and pseudo-globulin. Such fractions, therefore, must be looked upon as mixtures. Or if we look upon the serum proteins as one molecular structure in the serum, such fractions must be regarded as portions of that molecular structure which have so far resisted the disintegrating action of the salt; but which, by further action can be resolved into globulin and pseudo-globulin. And it may be further remarked that by precipitation at suitable concentrations fractions of material or portions of the original molecular aggregate may be obtained having any required solubility, and exhibiting a certain appearance of constancy. This is much more the case with mixtures of globulin and pseudo-globulin than with those containing albumin. The two former are much more closely connected at any rate in regard to their behaviour to salt, than are pseudo-globulin and albumin. As we have already seen, albumin can be removed comparatively readily.

I thus arrived at the general conclusion that there are two, and only two different proteins that are precipitable at half-saturation with ammonium sulphate; the historic water-insoluble globulin and the protein soluble in water corresponding to Hofmeister's pseudo-globulin. In addition to the precipitation differences between these two bodies, there are also certain differences in appearance. The soluble substance comes down in finer particles and has not the flocculence of the globulin precipitate. It is perhaps rather whiter and does not become discoloured so readily as globulin.

The Separation and Purification of Pseudo-Globulin.

We are now in a position to discuss the separation and purification of pseudo-globulin in greater detail, with a view of determining the limits of fractional methods, and the most convenient way of applying those methods to this particular case.

To obtain crude pseudo-globulin for further experiment, diluted ox-serum was taken; globulin and albumin got rid of at one-third and half-saturation respectively. After some two to three repetitions, a sample of pseudo-globulin can be obtained, giving no precipitate at one-third saturation, and leaving only traces of protein in the filtrate at half saturation. I will now describe the method of fractional precipitation I have hitherto employed in greater detail.

The crude protein, in this case pseudo-globulin, is dissolved in water and saturated salt solution is added until a precipitate begins to fall. This

is usually just after one-third saturation. In the early stages of the experiment a good precipitate is obtained at " one-third " saturation of the fraction. After the first few removals of globulin, however, this is no longer the case. The concentration of protein in the solution at this point may be 2–0·5 %. The readiest way of determining this is by a Kjeldahl determination of the total organic nitrogen, calculating the protein as containing 16 % N. The precipitate formed may be some 20 to 25 % of the whole quantity of the protein present. This is determined by comparing the opacity of the precipitate formed with that in a small quantity of the solution in which the protein has been completely precipitated by the addition of solid salt as has been described before. In using this method it must be remembered that the precipitate continues to fall gradually for some hours after the addition of a quantity of salt, so that only a rough determination can be made at the time of adding salt. The rate of fall of the precipitate appears to depend, to some extent, on the concentration of the protein : the diluter the solution the slower the fall. After the formation of the precipitate the solution is allowed to stand 24 hours. If the precipitate has sunk to the bottom, most of the liquid can be decanted. The remainder is filtered and the precipitate is redissolved. Some 35–30 % of this is then precipitated at about the same concentration as in the previous experiment.

This precipitate being some 9–6 % of the total protein present, is removed, and the filtrate is added to the main solution. The amounts withdrawn are gradually lessened as the experiment is repeated and may be tested for the protein it is desired to eliminate, in this case dialysis being used to detect globulin.

The process can be repeated indefinitely. When, however, the separation proceeds slowly, that is, when at each precipitation the proportions of the substances to be separated do not differ much in the precipitate and filtrate, it is expensive both of material and time. If the fractions are large the material is soon used up, and if small the process becomes very slow.

The amount of the fraction should correspond to some extent with the amount of protein present which it is desired to eliminate. As will be seen from the experiment quoted, some six or eight repetitions of this process will reduce the insoluble globulin in pseudo-globulin from 8–10 % to 2–3 %. The volume of the solution is increased at the end of each pair of precipitations. When it becomes inconveniently large it can be reduced by salting out the protein completely from a portion, dissolving the protein so obtained in a minimum of water, and returning it to the main solution.

In employing this method in the separation of the albumoses in some

cases I had no means of determining the progress of the separation, beyond that of noting the increasing solubility of the protein being purified and obtaining from successive fractions diminishing quantities of the more insoluble protein. Where hetero-albumose was being salted out there was the more independent test of precipitation by dialysis. This is the case in the present instance. This test, however, is only a solubility one, and cannot be relied on in the same way as some independent reaction. In the present case we have this latter: since, while globulin contains phosphorus, pseudo-globulin does not contain any. Globulin only contains about 0.1% P, so that in spite of the possibility of detecting very minute quantities of phosphorus the test is limited in its usefulness. It is sufficient, however, to show the general course of the separation, and to enable us to place a value on the solubility tests.

Hardy [1905] first pointed out that globulin contained phosphorus and that other fractions of serum-protein containing less globulin contained less phosphorus. The relationship of phosphorus to globulin is not quite simple as will be pointed out more fully later on. It is sufficient for present purposes to note that salting out operations and dialysis apparently leave the phosphorus content of globulin unchanged; while samples of pseudo-globulin can be obtained which, by the most careful tests, give no phosphorus.

For detecting and roughly estimating minute quantities of phosphorus the ammonium phosphomolybdate method of Neumann was used; the decomposition of the protein being effected by Bayliss and Plimmer's modification to avoid using more sulphuric acid than is necessary. Ammonium phosphomolybdate contains only some 1.6% P, and as very small quantities can be precipitated and detected, it forms, under proper conditions, a very delicate test. Further, owing to the way in which minute quantities of the precipitate fall, it is possible, simply by inspection, to estimate the amounts with rough accuracy.

Taking known quantities of a solution of sodium phosphate I have found that 0.005 mg. P can certainly be detected, sometimes 0.0025 mg. P. And if the conditions as to total volume of solution, quantities of reagents present, and heat used to effect precipitation, are maintained equally, the differences between such quantities as 0.005, 0.0075, 0.01, 0.015, and 0.02 mg. P, can be readily appreciated; and, by comparison, a rough estimate can be formed of the trace of phosphorus under consideration.

Good results were obtained with solutions differing slightly from Neumann's:

30 c.c.   Water.
30 c.c.   50 % ammonium nitrate solution.
30 c.c.   3 % ammonium molybdate solution.
Heat to 85° C.

Experiments were also made to determine how far Neumann's method could be used to estimate small quantities of phosphorus. Using a decinormal in place of a seminormal standard solution, I found that the method did not lose in accuracy until the quantities were below 0·2 mg. P, when the estimations became too high. Below 0·1 mg. P (which corresponds to 0·9 c.c. decinormal solution) the values were quite unreliable :

(1)	0·52 mg. P	gave	4·8 c.c.	N/10	solution	...0·539 mg. P
(2)	0·26 ,,	,,	2·5	,,	,,	...0·275 ,,
(3)	0·26 ,,	,,	2·5	,,	,,	...0·275 ,,
(4)	0·208 ,,	,,	2·1	,,	,,	...0·231 ,,
(5)	0·208 ,,	,,	2·0	,,	,,	...0·22 ,,
(6)	0·104 ,,	,,	1·1	,,	,,	...0·121 ,,
(7)	0·104 ,,	,,	1·0	,,	,,	...0·11 ,,
(8)	0·104 ,,	,,	1·15	,,	,,	...0·126 ,,
(9)	0·078 ,,	,,	0·9	,,	,,	...0·099 ,,
(10)	0·078 ,,	,,	0·8	,,	,,	...0·088 ,,
(11)	0·052 ,,	,,	0·65	,,	,,	...0·0675 ,,
(12)	0·052 ,,	,,	0·8	,,	,,	...0·088 ,,

For the experiments about to be described some three different samples of ox-serum (each of about one to two litres) were mixed. In order to determine the amount of phosphorus in the globulin of this mixture of serums, some globulin was separated and purified, first by salt precipitation, and then by acetic acid in the way previously described.

200 c.c. of 7th filtrate gave 1·2 c.c. N/10 NH$_3$.
200 ,, 8th ,, ,, 1·1 ,, ,,

The globulin was washed on the filter paper until no more acid was shown in the filtrates and then washed with alcohol and ether. It was dried at 110° to constant weight.

1·0965 g. globulin gave 1·2055 mg. P = 0·11 % (by Neumann).
0·732 ,, ,, 0·785 ,, = 0·107 %.
Mean = 0·1085 %.

Therefore, 1 mg. P indicates 0·921 g. globulin.

I. Crude pseudo-globulin, giving no precipitate at one-third saturation and containing traces only of albumin. To determine the amount of globulin contained in it phosphorus was estimated by Neumann's method.

2·172 g. gave 0·19 mg. P corresponding to 8·4 % globulin.

The fractional process described above was then carried out six times in succession ; large fractions were taken, some 28 % in the first instance and

35–40 % in the second. There was a marked increase in the solubility of each successive fraction, until, in the last fraction removed, I was unable, even after four sub-fractionations, to find any protein insoluble at one-third saturation. On the other hand, the fraction gave a distinct, though small, precipitate on dialysis. The experiment was terminated by salting out all the protein. To get rid of the small amount of albumin it was redissolved in water and thrice precipitated at half-saturation.

$$200 \text{ c.c. of 2nd filtrate gave } 2\cdot4 \text{ c.c. N/10 NH}_3.$$
$$200 \quad ,, \quad 3\text{rd} \quad ,, \quad ,, \quad 2\cdot3 \text{ ,,} \quad ,,$$

Thus albumin was eliminated.

On dialysis of a portion a good cloud appeared. The whole amount of protein was precipitated by the addition of alcohol in the cold (below $15°$ as advised by Mellanby) and washed with alcohol and ether. A portion was dried at $110°$ to constant weight and the phosphorus estimated:

(1) 0·4115 g. gave 0·0125 mg. P by inspection...2·8 % globulin.
(2) 0·442 ,, 0·015 ,, ,, ...3·1 % ,,

If we take a mean and allow 20% error, the amount of globulin in the sample is from $2\cdot3–3\cdot5 \%$.

The great contrast between the solubility in salt and the precipitability on dialysis was surprising and will be referred to later. This inconsistency was no doubt the basis of the observation by Freund and Joachim, who found that the fraction which gave no precipitate of globulin at one-third saturation gave a precipitate on dialysis so large that it was considered another substance.

II. Crude pseudo-globulin as in I. This experiment was on a much larger scale, and the process was repeated 36 times. The fractions were smaller than in I, being at first 20%, but dropping later to $12–15 \%$. The dilution varied from $1–0\cdot5 \%$ in the earlier stages to $0\cdot2–0\cdot1 \%$ in the later.

The salt solubility of succeeding fractions was noted, and a general agreement with the results in I was found.

After the ninth and twentieth fractions, the whole protein was salted out and dried. On each occasion it was found on re-solution that the solubility had decreased, and further that fractions taken immediately after re-solution were less soluble. The tenth fraction had the same solubility as the fifth, and the twenty-first as the fifteenth.

All the sub-fractions were tested by dialysis. It was sought by this means to follow the progress of the separation. The quantity of protein precipitated showed in general a decline. The decline was not a regular one, however. The first nine tests showed a steady decrease of substance. The tenth (after

drying) showed an increase, and from this to the seventeenth a slower and more irregular decline was noted. The twenty-fifth fraction showed no precipitate on dialysis, but the sub-fraction gave a cloud. The thirtieth fraction and sub-fraction remained clear. The thirty-first sub-fraction, however, gave a good cloud and the fraction a faint cloud. The thirty-fourth fraction and sub-fraction were again clear and also the thirty-fifth.

In general it was noted that when the fractions taken were small, the separation seemed to make very little progress. Observations were made as to the composition of the precipitates on dialysis, since in some cases they appeared out of proportion to the amounts of globulin that could be present. It was found that a considerable amount of pseudo-globulin was precipitated with the globulin, as the experiment became more advanced. The precipitate caused by the dialysis of serum or of the mixture of proteins obtained by one or two precipitations by " salting out " consists mostly of globulin, a certain proportion of pseudo-globulin and a small amount of albumin. The earlier fractions on dialysis gave such precipitates. In the later fractions, after the twelfth, it was found that on dialysis a large quantity of pseudo-globulin was precipitated with the globulin—in some cases an excess of pseudo-globulin. In one case where some 6–7 % of the protein present was estimated to be globulin, the precipitate was some 15–20 % of the protein present. Allowing, therefore, for 2 % globulin remaining in solution, the precipitate must have contained some 70–80 % pseudo-globulin, to 20–30 % globulin. Without attaching too much importance to these estimates, it is clear that considerably more pseudo-globulin than globulin was in this precipitate.

Precipitates of this kind remain insoluble, or nearly insoluble in water. They may be largely dissolved by the addition of salt, or better, a trace of alkali. In the latter case, if the solution be neutralised, no reprecipitation occurs, and further, if the solution be then dialysed there may be only quite a small proportion of the protein precipitated, or even none at all. This latter result could be attributed to traces of acid or alkali being left over after imperfect neutralisation. This was shown to be the case in many fractions containing small quantities of globulin. A portion dissolved in water and dialysed gave a small precipitate. Another portion which had been dissolved in dilute ammonia and then neutralised gave no precipitate on dialysis : nor did subsequent additions of traces of acid or alkali, nor prolonged dialysis, have any effect whatever in producing a precipitate.

The size and formation of the precipitate on dialysis appeared also to be connected with the rate at which the salt was withdrawn from the solution. In the majority of the experiments the precipitate reached its maximum in

24 hours; although there was then further salt in the solution, further dialysis produced no precipitate, even though continued many days. If, however, the solution was diluted four or five times with distilled water, a further precipitation was sometimes obtained.

It was also frequently noted that if pseudo-globulin containing a small proportion of globulin were dried, the precipitate on subsequent dialysis was increased. Both globulin and pseudo-globulin are rendered more insoluble by drying: by repeated drying both may be rendered entirely insoluble. So that the physical condition of pseudo-globulin would seem to be an important factor in inducing its precipitation with globulin on dialysis, and may have been one of the causes of the increase in the amount carried down with globulin already referred to.

It was also noted that, as the experiment advanced, a tendency to mechanical coagulation developed. This was chiefly noted in filtering, after a fraction had been precipitated. The protein in the solution then was just on the point of being precipitated. Unless the filtration was conducted slowly, small flakes and strings of protein appeared, which tended to adhere together at the end of the filter.

Towards the end of the experiment, also, the fractions were more affected by drying than in the early stages. Thus there is distinct evidence that the prolonged contact with salt induces slow physical changes in pseudo-globulin.

Only one intermediate observation of the amount of phosphorus was made. After nine fractions a portion was freed from salt, and precipitated by alcohol and dried.

$$0.347 \text{ g. gave } 0.01 \text{ mg. P by inspection......} 2.6 \text{ }^0/_0 \text{ globulin.}$$

This shows a rather slower rate of progress than in I.

Of the final substance 0.561 g. was tested for phosphorus with a negative result.

The physical changes noted during the experiment were:

1. A gradual increase of solubility in salt solutions which, after a time, appears to cease. In no case did the increase go so far that pseudo-globulin became soluble in half-saturated, or nearly half-saturated solutions (that is at ordinary dilutions, $5-0.2 \text{ }^0/_0$).

2. The substance gradually became more liable to pass into the insoluble or coagulated state.

In regard to the dialysis test it was found:

1. That the amount of pseudo-globulin carried down with globulin on dialysis increases as the experiment proceeds; the pseudo-globulin, after a time, being in excess of the globulin.

2. That if the substance be dried before dialysis a considerably larger precipitate results.

3. That the amount and constitution of the precipitate is also dependent on various chemical conditions.

In regard to the phosphorus estimations, assuming that 0·005 mg. P is the smallest amount that can be detected, the final result would mean that the pseudo-globulin might contain a quantity less than 0·00087 % P, but not more. This, together with the rather better result in III, where nearly a gram gave a negative result, is practically conclusive that pseudo-globulin does not contain phosphorus. We can, therefore, attribute the small and diminishing amounts of phosphorus found in the experiments to the presence of globulin, and so far as we can estimate the phosphorus we can estimate the globulin.

Experiments I and II, therefore, show:

1. That by the method described all but some 2–3 % of the globulin can be eliminated in some six or eight fractionations.

2. That the larger the fractions taken, the more rapid the separation. So that the method becomes too lengthy if used economically as regards material.

3. That both solubility tests as guides to the progress of the separation are fallacious.

As to the progress of the separation in II after the ninth precipitation, it is clear from the negative result in the final test for phosphorus, that most of the remaining 2·6 % of globulin is eliminated. It would seem probable that the elimination is a very gradual one.

In the albumose separation I relied on solubility tests, especially the salt solubility one. In no case, however, did the indications point to such a rapid conclusion of the separation as in I: nor were such large divergences as those between I and II met with. Although, therefore, there may have been some small alterations in solubilities which would tend to falsify results, it is hardly possible that it could have been so extensive as in the present case. In all respects the albumoses showed themselves more stable under treatment than the serum protein.

Finally, I tried a different method with a view of obtaining greater rapidity. In place of taking only a small proportion of protein at each precipitation, which is only efficient when a comparatively large proportion of globulin is contained therein, I tried the following plan which is based on the principle of dividing the protein into two equal parts at each precipitation.

III. Some 10 g. of crude pseudo-globulin which had been fractioned

four times on the method described above, and contained about 4% of globulin, were dissolved in 2,000 c.c. water. By the addition of solid salt about half the amount of protein as estimated in the way above described of comparing opacities was precipitated. The precipitate P_{-1} was dissolved in 2,000 c.c., and again by solid salt equally divided into P_{-2} and F_0. The filtrate F_{+1} was further divided equally as far as possible into P_0 and F_{+2} by the further addition of salt. At this stage, there were four divisions each containing about a quarter of the original protein. The process can be best explained by means of a diagram.

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                              O
              ┌───────────────┴───────────────┐
            P_{-1}                           F_{+1}
        ┌─────┴─────┐                   ┌──────┴──────┐
      P_{-2}      F_0=P_0             F_{+2}
   ┌────┴────┐  ┌───┴───┐        ┌──────┴──────┐   ┌────┴────┐
  P_{-3}  P_{-1}=P_{-1}  F_{+1}=P_{+1}      F_{+3}
      ┌────┴────┐    ┌────┴────┐    ┌────┴────┐   ┌────┴────┐
    P_{-2}    F_0=P_0   P_{+2}=F_{+2}   F_{+4}
       ┌───┴───┐   ┌────┴────┐   ┌─────┴─────┐   ┌────┴────┐
     P_{-1}   F_{+1}=P_{+1}   F_{+3}=P_{+3}   F_{+5}
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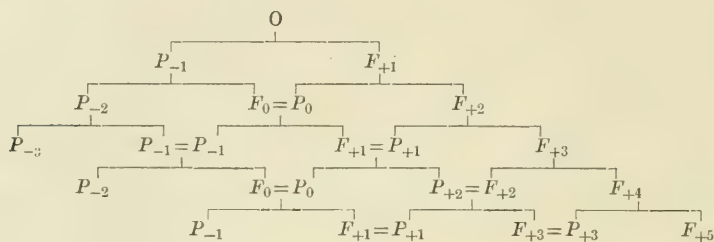
F_0 and P_0 were mixed being considered nearly of the same grade in purity; the precipitate being dissolved in a minimum of water and added to the filtrate. The mixture was then precipitated by the addition of solid salt as before and divided into P_{-1} and F_{+1}.

At each precipitation the globulin is divided into two portions, most going into the precipitate; so that the amount of globulin in the filtrates on the right F_{+1}, F_{+2} and F_{+3} is constantly diminishing. The process which involved a considerable number of precipitations was continued until a sufficiency of material of grades 3, 4, and 5 was obtained. It was found that an exactly equal division of the protein in any given experiment could not be obtained without an undue amount of time being spent. The method used was that already described of comparing opacities of precipitates. Owing, however, to the gradual fall of the precipitate which is the more accentuated the more dilute the solution, the precipitate cannot properly be estimated until twenty-four hours have elapsed. To save time, therefore, I endeavoured to allow for the extra fall on standing, by precipitating only some 35 or 40% as estimated shortly after adding the salt. This led to several inexactitudes and probably accounts, in part, for the slight inequality of the results.

The phosphorus estimations were all done at the same time, and compared with each other as well as with known amounts:

Parent substance 0·335 g. gave 0·015 mg. P...4·1 % globulin. (By inspection)
Third grade   0·511   ,,   0·0075   ,, ...1·3 %   ,,   ,,
Fourth grade   0·867   ,,   0·0075   ,, ...0·79 %   ,,   ,,
Fifth grade   0·883   ,,   negative, *i.e.* contains less than 0·5 % globulin.

The figures, allowing for the roughness of method of estimation, are sufficient to show that this method is more suited to eliminate small quantities than the other. It also has the advantage of economising time and material, as the precipitations, though numerous, can be carried on simultaneously, after the first few ; and all material in various grades can be labelled and kept ready for use at any time.

These estimations of phosphorus, restricted as they have been owing to the small amount present, are, I think, sufficient to establish the main point that the separation of these two proteins can be completed, or nearly completed, by means of fractional precipitations.

In regard to the practical point of the preparation of pseudo-globulin containing, say, not more than 1 % globulin, I do not think any very extensive procedure will be necessary. I found that in precipitating pseudo-globulin containing some 2–3 % of globulin with alcohol in the cold, that a small fraction could be obtained which was comparatively rich in globulin. I have not concluded my experiments on this point, but it is not unlikely that after a few fractions with salt have got rid of all but some 3 % or so of the globulin, that a large part of the latter can then be eliminated by a few fractions with alcohol.

The Preservation of Globulin and Pseudo-Globulin.

I have already pointed out that both globulin and pseudo-globulin on being dried, at any rate with salt, become more insoluble. To keep them without drying I have found that, by the addition of a little ether to their solutions in dilute saline, they will keep for considerable periods in stoppered bottles. The solutions should be well shaken with the ether.

I have samples which I have kept over a year which are quite clear and show no tendency to precipitate.

Relation of Phosphorus to Globulin.

In the preceding section I have assumed that globulin contained phosphorus as an integral part of itself. This was because throughout such reactions as there described the globulin always retains phosphorus. It is difficult to believe that if the phosphorus could be split off by salt, ammonia,

or acetic acid, that any except a trace would have remained with the globulin after the prolonged operations entailed by its purification. However, to obtain further assurance I estimated the phosphorus in a sample of crude globulin prepared from serum by four precipitations of salting out at one-third saturation, followed by two by acetic acid, after solution in ammonia.

1·4725 g. globulin gave 1·462 mg. P...0·099 %.

This is distinctly less than in the case of the more purified globulin although it had suffered less than half the number of precipitations.

I have already quoted (page 507) the phosphorus determinations in one sample of purified globulin. Another sample from ox-serum was analysed:

0·5495 g. globulin; 0·575 mg. P...0·105 %,

a very similar result.

Treatment of dried globulin with alcohol or ether gives a yellow, rather fatty extract, which contains phosphorus. This extract is only separated from globulin with some difficulty. I have tried various means; treatment of the finely powdered globulin in a Soxhlet apparatus with ether; prolonged shaking with alcohol and ether; and boiling with alcohol, after Plimmer. The latter was the most effective; an extract could generally be obtained by its means from samples which had been previously treated in other ways. I found, however, that though most of the extract could be obtained after some eight to ten hours boiling, that further small quantities could be got by more prolonged boiling. In no case could I get anything like a phosphorus-free globulin. I quote two experiments in which the globulin, well powdered, was boiled with successive portions of alcohol for periods of seven hours, until no more extract was obtained.

(1) 0·5265 g. globulin gave ext. cont. 0·3096 mg. P ; 0·056 %. Boiled 21 hrs.
(2) 0·68625 g. ,,   ,,   ,,   0·3207   ,,   ; 0·048 %.   ,,   35 ,,

Thus about half the original phosphorus is got rid of in the extract. In previous experiments in which prolonged shaking (200 to 300 hrs.) was adopted, not more than 30 or 35 % of the phosphorus was eliminated. I also shook solutions of crude globulin and diluted serum directly with ether. In the former case the results were negative, though in one or two experiments minute traces of an extract similar to that looked for were obtained. With serum, no trace of the extract looked for was found, but an orange pigment was extracted without much difficulty. From pure or nearly pure pseudo-globulin no extract could be obtained by any of these means, though

small quantities were found in samples containing 3 or 4 % of globulin, as would be expected. The amount of extract obtained from globulin measured by weight was some 8–10 % of the globulin treated.

In view of the difficulty of completely extracting solids by liquids and especially the very slow action of the extracting liquids in this instance, it is tempting to suppose that the whole of the phosphorus in globulin belongs to some lecithin-like body or bodies which would appear closely connected with the globulin, but not part of the molecule. These would then amount to no less than 15–20 % by weight of the globulin. On the other hand the present facts do not warrant us in accounting for more than half the phosphorus in this way.

SUMMARY.

1. There are two proteins of ox-serum insoluble in half-saturated solutions of ammonium sulphate, saturated magnesium sulphate, or sodium chloride; the historic water-insoluble globulin, and the water-soluble body pseudo-globulin.

2. These two bodies cannot be split up further by means of fractionation with salt and water.

3. Intermediate fractions are shown to be mixtures of these two bodies.

4. Globulin contains, or is closely associated with phosphorus, rather more than 0·1 mg. P % being found. About half this belongs to a fatty, lecithin-like body which amounts to some 8–10 % of the globulin freed from pseudo-globulin. Apparently no part of this body is detached from globulin through prolonged treatment with acids, alkalies, or salts.

5. Pseudo-globulin does not contain phosphorus.

6. Repeated precipitations of globulin at constant volume finally give filtrates in which the amount of protein is constant. Globulin, presumably, can thus be freed from pseudo-globulin.

7. Pseudo-globulin can be freed in a similar way from albumin, the separation being considerably easier.

8. Pseudo-globulin can also be freed, or nearly freed, from globulin by suitable methods of fractional precipitation.

9. The presence of phosphorus has given us an independent test for following the progress of the latter separation. The result has been to establish the validity of the general method of fractional precipitation in this case, to give important indications as to the most suitable method to use, and to estimate the value of the solubility tests.

REFERENCES.

Burckhardt (1883), *Archiv f. exp. Path. u. Pharm.* **16**, 322.

Freund u. Joachim (1902), *Zeitsch. physiol. Chem.* **36**, 407.

Fuld u. Spiro (1900), *Zeitsch. physiol. Chem.* **31**, 132.

Hammarsten (1878), *Pflüger's Arch.* **17**, 413.

Hardy [1905], *J. Physiol.* **33**, 330.

Haslam (1905), *J. Physiol.* **32**, 267.

—— (1907), *J. Physiol.* **36**, 164.

Heynsius (1869), *Pflüger's Arch.* **2**, 1.

—— (1876), *Pflüger's Arch.* **12**, 549.

Kander (1886), *Arch. exp. Path. u. Pharm.* **20**, 411.

Marcus (1899), *Zeitsch. physiol. Chem.* **28**, 559.

Mellanby, J. (1907), *J. Physiol.* **36**, 288.

Panum (1851), *Virchow Arch.* **3**, 251.

—— (1852), *Virchow Arch.* **4**, 17 and 419.

Pick (1902), *Beiträge* **1**, 351.

Porges u. Spiro (1903), *Beiträge* **3**, 277.

Reiss (1904), *Beiträge* **4**, 150.

Schmidt, A. (1862), *Müller's Arch.* 429.

Wiener (1911), *Zeitsch. physiol. Chem.* **74**, 29.

Zimmerman (1854), *Müller's Arch.* 377.

L. THE CHEMISTRY OF THE LEUCOCYTOZOON SYPHILIDIS AND OF THE HOST'S PROTECTING CELLS.

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INTRODUCTION.

The life-history of the Leucocytozoon Syphilidis has been fully described by McDonagh [1913, 1, 2]. The present communication represents an attempt to elucidate the micro-chemistry of the organism in question.

The first consideration will be given to the physical and chemical properties of the staining reagents used, then to the characters of the syphilitic bodies as evidenced by staining *in vivo*; the same by the staining of fixed specimens, in which the protoplasmic and nuclear portions will be dealt with separately. Reference will then be made to some physical characters of the various cells, and attention given to some special chemical features of the syphilitic bodies and of the host's protecting cells. Finally, the physical and chemical characters of the lecithin-globulin complex will be mentioned.

THE PHYSICAL AND CHEMICAL PROPERTIES OF THE STAINING REAGENTS USED.

In all micro-chemical investigations it is important fully to consider the chemical and physical properties of the staining reagents before drawing any conclusions as to the chemical nature of the structures under consideration. The dyes as a group are almost entirely colloidal in nature, that is to say, they do not readily diffuse through animal membranes. Their colloidal nature is also emphasised by their high molecular weight and absence of crystalline forms. In solution these dyes form typical colloidal suspensions, the size of the particles varying in different cases; consequently we notice that some stains appear quite homogeneous, whilst others show the

well-marked characters of suspensions. Further, many dyes are removed by filtration and may be separated by this means, as, for example, methylene blue occurring in the urine after subcutaneous injection.

Owing to their colloidal nature the small particles of a dye in suspension become electrically charged, and we may for convenience divide up dyes into negatively and positively charged colloids. The negatively charged colloidal particles of a dye will, therefore, show electrical migration to the anode, whilst positively charged colloids will travel towards the kathode. It follows from this also that on mixing a positive dye with a negative dye the charges will be neutralised and the resulting colloidal mixture will then exist in an uncharged condition; *vide* the eosin-methylene blue mixture, the former being negatively charged whilst the latter is positively charged.

But the one great feature of all dyes is that they exhibit the phenomenon of adsorption (Bayliss). By adsorption we mean a "combination" between two substances which is not strictly in the nature of a chemical union, that is to say, in which there is no direct proportionality between the concentration of the solution and the amount adsorbed. There is some kind of physico-chemical affinity between the bodies adsorbed, and those which take them up, but this affinity is more in the nature of a mechanical than a true chemical affinity. To take an example, if a series of solutions of Congo red of varying concentrations be taken, and the same amount of filter paper be placed in each, a part of the dye is taken up, but in relatively larger proportions in the more dilute solutions of the dye.

There are, however, instances where the combination of the dye with the material acted upon is in the nature of a true chemical combination, but these are exceptions rather than the rule, *vide* the staining of nuclei with rongalit white.

A third and most important factor comes into play in the processes of histological preparations, namely, the presence of electrolytes. The dyes as a whole are very sensitive to electrolytes in regard to their adsorption properties and the effect appears to be proportional to the degree of their colloidal nature. Electro-negative dyes, like Congo red, are increased in adsorptive power by the addition of kations, such as lithium, potassium, sodium, ammonium, magnesium and calcium. On the other hand, anions such as hydroxyl, acetate, chloride, oxalate, sulphate and phosphate depress adsorption. With electro-positive dyes, such as toluidine blue, the reverse holds good, namely anions facilitate and kations depress. The effect of electrolytes, however, is much more marked in the case of kations than it is with anions. The salts of the heavy metals which form positively charged

colloidal hydroxides, for example, iron, have a very powerful effect on the adsorption of electro-negative dyes, and in every case the ion promoting adsorption of the dye is carried down with it.

If we turn now to histological preparations, perhaps we may in the light of these observations be able to interpret the changes induced in the act of staining. In the living cell the substance to be stained has a negative charge, as the reaction of the tissues is always on the alkaline side of neutrality, and we know that protein solutions in an alkaline medium are always negatively charged; it follows, therefore, that living cells will take up basic dyes and that electrolytes are not essential for the process. When the cells die the electrolytes attached to the protein constituents of the cell are split off, with the result that the cells now readily take up acid dyes. Further, we know that the fixation of a dye is facilitated by heat, and this fact has been made use of in Altmann's method of acid-fuchsin staining. Mayer has also shown that the Nissl bodies of nerve cells have their affinity for basic dyes destroyed by previous treatment with neutral salts; an observation which further emphasises the importance of electrolytes in the staining act.

Let us now turn our attention to the observations upon the syphilitic parasite and see whether the results obtained can be interpreted in the light of these views of staining. In the first place, we will consider the two principal stains used, viz. pyronin and methyl green. Both these dyes are positively charged colloids and in consequence will be facilitated in their staining by anions, particularly hydroxyl and sulphanion. The pyronin will, therefore, act as a basic dye and this explains why it is precipitated by nigrosine and also diamine green, but not by diazine green. Making use of the chemical characters of the dyes used and reviewing all the results obtained in this light, it is obvious that much more useful and valuable knowledge of the micro-chemistry of the cells under investigation can be ascertained. The specific staining properties of the syphilitic parasite will be again referred to and all we would point out here is the importance of regarding pyronin as a positively charged colloid, and influenced by anions.

THE CHARACTERS OF THE SYPHILITIC BODIES WHEN STAINED "IN VIVO."

The simplest and best way to use a reagent for vital staining is to spread a solution on to a slide free from fat and alkali and then allow it to dry in the air. The film should be made just before it is required and not kept several days. The material to be examined is placed upon a cover slip and

the latter so adjusted to the slide that no air remains in between and so that as little as possible of the fluid to be examined exudes at the sides. Examination is possible until the fluid dries, which takes several hours. Ringing the cover slip with wax greatly facilitates the process. The slides used for hanging drop preparations are useless, but a warm stage may sometimes be advantageously employed.

We have used all the stains which have been from time to time advocated, but have not obtained the results which one might be supposed to get from reading the literature about them; therefore, it might be as well to state that neutral red, neutral violet, Bismarck brown, auramine, diazine green, malachite green, tropaeolin 00, and Congo red do not give good vital staining. Owing to the use which is now being made of the azo-dyes in determining the functional activity of certain cells in the body, we attempted several for our method of staining *in vivo*, but by no means with success, because they have such feeble staining properties and are general protoplasmic stains without possessing affinity for certain structures; moreover, they do not possess metachromatic properties. The dyes which gave the best results were aqueous solutions of borax-methylene blue and polychrome-methylene blue; brilliant crystal blue, Nile-blue sulphate and alcoholic solutions of toluidine blue, thionine and azure II. The disadvantage of the alcoholic solutions is that they must be diluted well before use as crystals so readily form, and this is especially the case with thionine. Azure II stains deeply, but unfortunately has no metachromatic properties. The intracellular stages are perhaps better depicted by the alcoholic stains, but taking everything together the results are not so good as when the aqueous solutions of either borax-methylene blue or brilliant crystal blue are used: and, of the last two, the former is superior. The metachromatic properties of borax-methylene blue are dependent upon the basic sodium biborate, which acts upon methylene blue by producing methylene violet which, as a basic dye, shows affinity for acid substances, and methylene red which is an acid dye and so shows affinity for basic substances.

We tried various other bases with methylene blue with the hope of obtaining greater metachromatic action by substituting a base of higher valency for the borax, for which purpose we employed colloidal aluminium hydroxide. The methylene blue remained unaltered, no doubt owing to the fact that the aluminium hydroxide did not contain sufficient free hydroxyl ions and was in itself an unstable colloid and therefore had no action upon the positively charged methylene blue.

Borax-methylene blue, when freshly prepared, has practically no

metachromatic action, but this property increases the longer the stain is kept, until finally the methylene red becomes the stronger dye and stains the cells just as the methylene violet does. Borax-methylene blue appears to be at its best when it has been kept for a year or two. As the methylene red scarcely comes into play in the fresh solutions, no harm is done by adding 0·1 g. eosin to 100 cc. borax-methylene blue, as a true chemical compound results. The eosin picks out the granules in the polymorphonuclear leucocytes, also stains brilliantly the eosinophile granules, but not one of the stages of the syphilitic organism. When we had learnt that the syphilitic organisms contained lecithin in the form of a lecithin-globulin complex, and being aware of the affinity that this complex shows towards dextrose, it struck us that it might be possible to increase the staining properties of the organism by adding dextrose to borax-methylene blue. Although the dextrose did not carry the colloidal dye particles to the cells in question, it nevertheless was taken up by every cell which contained the lecithin-globulin complex, since the protoplasm of such cells swelled and absolutely refused to stain, but, owing to the swelling, were as easily discernible as if they had stained, consequently the plasma cells and syphilitic bodies could be well studied, as their nuclei were not prevented from staining. Witnessing the act of impregnation in a dextrose-borax-methylene blue specimen, we were able to compare it with what we had previously seen. The nucleus of the female appeared to float about, surrounded as it was by the clear ring of unstained protoplasm; when first seen one end of the spirochaeta had already entered, as one extremity was fixed to the nucleus; the spirochaeta was also thickened (due to the dextrose). The one end of the spirochaeta remained fixed to the female nucleus and, in spite of knocking against all the other cells in its progress, it remained attached to the same spot, but did not enter any further. Suddenly the female cell stopped and the spirochaeta pallida vanished inside, but 55 minutes had elapsed before this happened. No further change was noticed in the female cell, as it had not stained very well, but about four minutes later it became very active and discharged a clear non-staining polar body which seemed to be emitted with some force. A few seconds later another clear polar body was extruded and then the female cell came to a standstill again. It is possible that the dextrose made these polar bodies appear bigger than they really were, as each was certainly 2–3 μ in diameter.

From what has been stated it will be easily seen that a description of the syphilitic organism *in vivo* from its reactions will entirely depend upon the characters of the borax-methylene blue which is used, and as impregnated

female cells do not stain with eosin or the methylene red of freshly prepared borax-methylene blue, the increase in the basicity resulting from impregnation cannot be very great. It is far more probable that no change in reaction occurs and that the reason for staining with methylene red, a fact which we have frequently observed, is due to an increase in the reducing action, as will be shown later.

The sporozoites may remain for some time unstained or stain immediately a dense violet. The intracellular phases stain late and the early ones show an affinity for the methylene violet moiety, whilst the late ones, viz. the coils, take up the methylene red. The females before fertilization remain unstained except their chromatic network and blepharoplasts which stain immediately with methylene violet. The spirochaeta pallida stains pink and when it has impregnated a female cell and the whole cell has come to a sudden standstill, a pink diffuse stain comes over the cell like a mantle. The sporozoites while in the spore cysts show a greater affinity for methylene red than methylene violet, and some spore cysts are seen which stain distinctly metachromatically.

In staining *in vivo* with a negatively charged colloid it follows that alkaline basic dyes will react best, and this has been found to be the case; and the reason why neutral red, neutral violet, Bismarck brown, auramine, diazine green, malachite green, tropaeolin 00, and Congo red were found not to give good results is simply the fact that they are negatively charged dyes and therefore cannot stain cells which contain colloids in solution with a negative charge existing in a medium on the alkaline side of neutrality. It follows that good staining can only be obtained by using dyes with a positive charge, hence the reason why borax-methylene blue and polychrome-methylene blue serve so admirably, as it is only under such conditions that adsorption can come into play.

Summary. Basic stains are the most suitable for *in vivo* work, and of these borax-methylene blue is the best. Owing to the presence of a lecithin-globulin envelope the syphilitic bodies can be made to stand out clearer by adding dextrose to the stain. The varied affinity shown by the different bodies on the one hand for methylene violet and on the other hand for methylene red is due to the prevalence of a substance which has strong reducing properties (lecithin-globulin) and not to a change in the reaction.

THE CHARACTERS OF THE SYPHILITIC BODIES WHEN STAINED IN
FIXED SPECIMENS.

Both Pappenheim and Martin Heidenhain explained the specific action of methyl green for chromatin as being due to the breaking down of the weak basic salt by the strong nucleic acid radicle; on the other hand the nucleus did not stain with pyronin which they regarded as a stronger basic salt. As a matter of fact methyl green, being a triamino-stain, is by far a stronger base than the diamino-stain pyronin, the basicity of which is also diminished by its O-ring formation. Furthermore, acetic acid increases methyl green staining and if acids combine with the free amino-group of the salt, acetic acid would have done this before the nucleic acid got a chance. Therefore, the explanation of its action, which had held sway for some years, cannot be the correct one.

A stain which had been largely used by Unna, namely rongalit white, was found to resemble methyl green in many respects and was known only to stain the oxygen positions of the tissues.

Rongalit white is the leuco-base of methylene blue which is prepared with sodium sulphite and formalin. It is therefore a colourless and basic mixture and the methylene blue is only brought out as a dye in the presence of oxygen. As rongalit white stains the nuclear part of the cell, Unna concluded that methyl green also picked out the oxygen foci of the tissue and was, therefore, a reduction-sensitive stain.

By a series of experiments Unna showed that methyl green was far more sensitive to reducing substances than methylene blue and that malachite green came in between, but that such reducing agents as grape sugar and hydroxylamine were without effect, i.e. did not decolourise methyl green. Another difference between methyl green and methylene blue and malachite green was that the leuco-bases of the last two could be reconverted into their coloured bases by the addition of hydrogen peroxide, but not so methyl green.

Until Unna enunciated his theory of staining by oxidation and reduction we were under the impression that staining depended upon reaction; in other words, that acid substances stained with basic dyes and were therefore termed basophilic, and basic substances stained with acid dyes and were therefore termed acidophilic. No doubt in part this conception is correct, but there is also no doubt that it was carried very much too far. After all, fixed protoplasm is an amphoteric substance, i.e. it can act as a base or an acid; for instance, the protoplasm of plasma cells stains well with acid

fuchsin, which is an acid dye, or with pyronin, which is a basic dye. Although it stains with both, it does so better with the latter than the former; therefore under ordinary circumstances, it can be stated that protoplasm, using the word in a very general sense, prefers to act as an acid.

From McDonagh's description of the Leucocytozoon Syphilidis and the coloured plates which illustrate his article [1913, 2], it will be seen that by using Pappenheim's stain (a mixture of pyronin and methyl green) the syphilitic bodies stain with pyronin, but differ from all other cells in that the nucleus also apparently stains with pyronin and with a much deeper red than the rest of the cell. Working on the reaction hypothesis, or on the electrolytic theory, one must assume then that the protoplasm and especially that of the nucleus of the syphilitic organism is strongly basophilic and negatively charged, but we have already shown it to be partly acidophilic from our *in vivo* examinations, when we pointed out that certain phases showed an affinity for methylene red. We have then a paradox and a solution to the problem can only be found if we adopt Unna's theory of oxidation and reduction.

Methylene violet, like methyl green, is a reduction-sensitive dye, although not to the same degree, and the reason why certain phases stain with methylene red is not because the protoplasm is acidophilic, but because it has reducing properties, and as methyl green is far more sensitive than methylene violet, every phase stains with pyronin, while only in those in which the reducing action is greatest is the affinity of methylene violet for nucleic acid overcome, with the result that the reducing substance stains with methylene red. In fixed specimens this reducing substance does not stain very readily with acid dyes, should a basic dye be present as well, because if pyronin is supplanted by acid fuchsin, most of the nuclei of the syphilitic organisms stain with methyl green; therefore, this characteristic pyroninophile substance of the syphilitic organisms is a strong reducing agent, is basophilic, and, accordingly, negatively charged according to the electrolytic theory; but the action of its electric charge is overshadowed by its reducing action. Therefore, we have another extremely important factor coming into play in the act of staining.

Seeing how sensitive a stain methyl green is, it at once appears obvious what caution must be taken in choosing the most suitable fixing reagent, and that any fixing reagent which robs the nucleus of its oxygen will naturally prevent staining with methyl green, and will also alter the action of the medium. Fixing tissue for 24 hours in a 1 % solution of platinum chloride increases the capacity of the nuclei for methyl green, but it is an

expensive solution and does not give such good results as some other fixing reagents. Mercuric chloride has the disadvantage that the sections may stain unevenly, since it only increases the capacity for methyl green staining in the situation where it remains and diminishes it in those situations where it is reduced by the tissue. If mercuric chloride is employed in an alcoholic solution as a fixing reagent, quite good sections may be obtained with Pappenheim's stain, and the effect is enhanced if a little acetic acid is added to the mixture. Chrome salts render staining with pyronin and methyl green impossible. Osmic acid alone or in conjunction with other acids diminishes the receptivity of protoplasm to most dyes.

Formalin, owing to the formic acid which it so frequently contains as an impurity, not only diminishes the staining properties of protoplasm, but markedly reduces the power of the nuclei to stain with methyl green. Including other fixing reagents which are seldom used and are not available for obtaining satisfactory sections with Pappenheim's stain, we are left only with alcohol, and from several experiments we have undertaken we are firmly of the opinion that alcohol is far and away the finest fixing reagent we possess at present, as it allows staining with most of the stains in general use, it fixes by coagulation, and forms no chemical compound with the cells and is, therefore, extremely well adapted for the purpose of micro-chemical research when fresh sections are not employed.

We either employ absolute alcohol or 50% alcohol; the former causes shrinking of the intercellular tissue but not so much of the individual cells and its main advantage is that coagulation is immediate. The moment the tissue is removed from the body it is placed at once into absolute alcohol and allowed to rest on wool—whenever alcohol is used a pad of wool should rest on the bottom of the bottle so as to allow the alcohol to remain the same strength throughout while the water extracted from the tissue sinks through the wool; this simple device means also a great saving of alcohol. The tissue remains for 12 hours in absolute alcohol and can then be changed into two further lots of absolute alcohol for 12 hours each or be put back to 50% and gradually taken up in the usual way. Cedar wood oil is used for clearing, as it does not harden the tissues to the same extent as xylene, and before it is put into wax two changes of xylene are used for 1–2 hours each, as wax penetrates better after the tissue has been through xylene. The wax used is a mixture of:

Paraffin (melting point 60° C.)	...	$8\frac{1}{2}$ parts.
Stearin	... ...	1 part.
Wax	... ...	$\frac{1}{2}$ part.

The melting point of the prepared article is 53° C. and the tissue is left in this, changed three times, for about six hours altogether. The whole of the secret of getting good paraffin sections is to be absolutely certain that the tissue is fully dehydrated.

The tissues can also be fixed in 50% alcohol, allowed to remain in the solution for 24 hours and then for 24 hours each in 70% and 90% and 12 hours each in three changes of absolute alcohol, and so on as before. There is not so much shrinkage of the tissue, and most excellent Pappenheim-stained sections may be obtained; but, owing to the solubility of certain substances in 50% alcohol, this method cannot take the place of absolute alcohol in micro-chemical research.

For some tests of minor importance celloidin sections are preferable, but for general purposes we much prefer paraffin, as thinner sections can be obtained; they can be more easily fixed to the cover slips, and one does not have to go through the troublesome procedure of removing the celloidin, which is necessary when aniline dyes are being used, owing to the intense avidity which celloidin has for many of them.

If Pappenheim's stain is to be used we proceed as follows: Roughly two parts of a saturated aqueous solution of pyronin are mixed with one part of a saturated aqueous solution of methyl green immediately before use, or until the mixture assumes a red-purple colour. In this stain the sections may be left from 5 minutes indefinitely as overstaining is impossible. After being in the stain the sections are transferred to a freshly prepared distilled water solution of resorcinol, which is just used for washing off the stain, i.e. about a minute, and then they are put into a freshly prepared absolute alcoholic solution of resorcinol and kept in until all the superfluous stain has come away.

These resorcinol solutions are absolutely essential, as they act as mordants and we regard mordanting after staining as superior to Unna's method, which consists in adding carbolic acid to the stain, which enables the stain to be prepared and always ready for use. The stain is sold under the name carbol pyronin methyl green. The great disadvantage of the ready prepared stain is that the pyronin comes out too quickly in the dehydrating process, while if resorcinol is used this is not the case. The amount of resorcinol crystals used in the first watch glass is about 0·3 g. and in the absolute alcohol watch glass, just double the quantity. From the resorcinol the sections go through three changes of absolute alcohol, two of xylene, and are then mounted in balsam.

As ethyl alcohol may abstract the pyronin stain from the sections, clearing fluid may be used to take its place, such as chloroform, lavender oil or

bergamot oil. Clove oil should never be employed owing to its powerful reducing action.

Xylene fortunately acts indifferently, but Canada balsam in time, owing to its reducing action and being an acid, destroys the staining effect; dammar dissolved in xylene would form a better medium for preserving the section. For a year or two, or even longer, sections stained in the above method and mounted in Canada balsam show a much sharper contrast of colour; the pyronin stands out clearer, the orange colour of the mast cells is more distinct, but the methyl green staining is somewhat weaker, and as time proceeds it is the methyl green stain which first disappears. Some sections prepared seven years ago are as good as and in many respects, owing to increase of sharpness, better now than then.

In a Pappenheim-stained section the protoplasm of the groundwork and connective tissue cells stains a rose pink and has a finely granular appearance, whereas the protoplasm of the plasma cells stains a clear red. All nuclei stain green, the nucleoli a brilliant red, the mast cell granules orange and all bacterial and protozoal bodies red. The protoplasm of the syphilitic bodies stains a rose pink to red, and the nuclei a deeper red. The difference in the rose pink to red of the protoplasm being most marked in the female cells is dependent upon whether they have been impregnated or not, as the fertilised female cells and zygotes always stain deeper. At first sight one might conclude that the supposed nuclear part of the syphilitic organism stains deeply with pyronin, because it contains no nucleic acid; but we have endeavoured to prove that such a surmise is incorrect.

We added acetic acid to the alcohol in which the tissues were fixed in the proportion of 1 cc. glacial acetic acid to 75 cc. either absolute or 50% alcohol, with the hope that if any nucleic acid was present the acetic acid would precipitate it. When the sections were stained we found that the general pyronin staining had not been interfered with, that the methyl green staining was strongly intensified, and that some of the nuclei of the syphilitic bodies stained a brilliant green, proving at once that they contained nucleic acid. The addition of acetic acid in the alcohol used for fixing, for ordinary staining with pyronin and methyl green gives better results than when alcohol is used alone, owing to the fact that apart from the nucleic acid being precipitated, the swelling action of the acetic acid on the cells is counterbalanced by the shrinking action of the alcohol and *vice versa*. For special staining, as when the demonstration of micro-organisms is required, alcohol alone is the one and only fixing reagent.

As methyl green is one of the ingredients of Ehrlich's triacid stain and

the red dye, acid fuchsin, is an acid dye in contradistinction to pyronin which is basic, we stained some sections with this mixture, with the result that the protoplasm of the syphilitic bodies stained red, while the nuclei stained green, another proof that the nuclei contain nucleic acid. We can, therefore, assume that there is some substance either in or over the nucleus of the syphilitic parasites which is a strong reducing agent, as it prevents the methyl green from getting at the nucleus and that it prefers basic to acid dyes.

Our next step was to try to determine the reducing action of this substance and also its degree of solubility in various reagents.

Reducing action. (*a*) Sections were stained for 1–2 minutes in a freshly prepared 1 % solution of potassium permanganate, then washed in water, decolourised in oxalic acid if overstained, dehydrated and mounted in balsam. All protoplasm has a reducing action and consequently stains brown with potassium permanganate while the nuclei remain unstained. The protoplasm of the syphilitic bodies has a greater reducing action than ordinary grano-plasma, but the nuclei do not stain and can be counterstained with methyl green; therefore, the reducing body has not a very marked action on potassium permanganate.

(*b*) Sections were placed for 5 minutes in a mixture of equal parts of a 1 % solution of ferric chloride and a 1 % solution of potassium ferricyanide. It is imperative that these two solutions shall only be mixed immediately before use, as if allowed to stand a blue precipitate is slowly formed. The reducing action of the granoplasma converts the ferricyanide into ferrocyanide with the result that where the reduction is greatest a beautiful Berlin blue colour is formed in the presence of the ferric chloride. Ordinary granoplasma has a weak reducing action and so stains green; the granoplasma of plasma cells and the syphilitic bodies have a stronger reducing action and so stain darker, while the nuclei of the ordinary cells do not stain, except some of the "nuclei" of the syphilitic bodies, which stain a faint Berlin blue colour. Red blood corpuscles also give the Berlin blue reaction and so do the aminoplasma cells, both to a more marked degree than the nuclei of the syphilitic bodies. Ordinary nucleoli have likewise a reducing action on ferric ferricyanide.

There is something quite peculiar in the appearance of the syphilitic bodies stained with potassium permanganate and ferric ferricyanide, because in the former, when counterstained with methyl green, the nucleus appears smaller than it really is; there is less to take the methyl green. In both it appears irregular, and scattered here and there about the nuclei are small non-staining transparent areas.

We will deviate somewhat from our course here and dwell upon the aminoplasma cells.

The aminoplasma cell is a form of plasma cell which Unna has called, from his examinations thereof in fixed specimens, Hyaline plasma cell. The term " Hyaline " rather suggests some relationship to cartilage, although it is very largely used for substances of which the observer has no knowledge. Now, hyaline cartilage is a strongly basophilic substance owing to its chondroitin-sulphuric acid radicle and therefore possesses great affinity for methyl green. Unna's hyaline plasma cells are, on the other hand, acidophilic and contain no acid radicle; furthermore, they have very strong reducing properties and so cannot stain with methyl green and, as we shall show presently that this reducing action is due to tyrosine, we consider that the best name for them is aminoplasma cells.

The cell is frequently to be met with in syphilitic material, but it is also to be found in any very chronic inflammatory lesion, viz. Rhinoscleroma and Ulcus Molle Serpiginosum. In in vivo specimens an aminoplasma cell is apt to be mistaken for a zygote, owing to the affinity which both have for methylene red, but the distinction becomes clear when it is borne in mind that the former may vary in size from 7–14 μ or more in diameter, that it may have no nucleus, that the nucleus stains homogeneously with methylene violet, that it may be situated in the centre of the cell or at the periphery and that it sometimes possesses the power of motion and may be extruded and finally excluded from the cell altogether. In the aminoplasma cells also are generally to be seen dots, masses or strands situated anywhere and irregularly scattered about the cell, which have no connection with the nucleus, although they stain deeply with methylene violet.

In fixed specimens the appearance of these cells is very different and instead of being round, homogeneous cells, they are often irregular in shape and divided up into irregular sized loculi or balls of protoplasm, many of which become loose and scattered about in the tissue. These balls stain with safranin, and acid fuchsin, and give a Berlin blue reaction with ferric ferri-cyanide. They do not stain well with pyronin, but in some specimens strands of protoplasm are to be noticed in between the loculi which do stain with pyronin. The strands are no doubt the same as the dots, masses and strands which were described in the in vivo method as showing an affinity for methylene violet.

These ballooned plasma cells have in some cases lost their nuclei, whilst in others the nucleus is lengthened out and fits one apex of the cell like a cap does the head, and not infrequently sends stringlike processes down over

the cell protoplasm. These cells are no doubt degenerated cells, because the protoplasm gives amino-acid reactions and in the most degenerated cells the nucleus gives the histone reaction and fails to stain with methyl green.

From what has been said it will at once be seen that the syphilitic bodies bear points of resemblance to the aminoplasma cells, but that they differ in the very striking point that the most reducing part of the syphilitic body stains deeply with pyronin, whilst the most reducing part of the aminoplasma cell stains faintly with pyronin, and, as we have shown that the reducing substance of the former is basophilic, it at once appears obvious that that of the latter is more acidophilic.

The Berlin blue formation is a fixed chemical process between tissue and reagent, since, although the colour can be caused to vanish with alkalies, it immediately returns on the addition of an acid, and, as the feeble reduction areas are more quickly decolourised than the firm Berlin blue areas, weak alkalies may be used to decolourise the former and the protoplasm of the cells can then be counterstained with an aniline dye.

(c) The third reaction we tried was with tetranitrochrysophanic acid $(C_{15}H_6O_4(NO_2)_4)$. It is a crystalline product obtained from chrysarobin which is dissolved in acetic acid and treated with nitric acid. The reagent is insoluble in water, and owing to the reducing power of ethyl and methyl alcohol it has to be dissolved and kept in chloroform or xylene.

After staining for 10 minutes the sections are returned to chloroform, put through three changes and through xylene, and then mounted in balsam. Weak reducing agents stain a pale rose red, strong reducing agents stain red. The protoplasm stains pale rose red, nuclei remain unstained, syphilitic bodies stain a deeper red, but the contrast is not so clear as in (a) and (b).

In tissues there are four chief classes of bodies:

    1.   Proteins.   2.   Carbohydrates.   3.   Fats.
    4.   Cholesterol, lecithin and allied lipoids.

All four groups possess reducing properties in varying degrees, but the second we can rule out in the present discussion as will be shown later. Therefore, the reducing substance must be a protein, a derivate of a protein, a fat, or a lipoid. Speaking generally, pure proteins, fats (olein excepted) and lipoids are not strong reducers, but derivates of proteins are, especially the amino-acids, and here is appended a list of amino-acids with their action on potassium permanganate and the ferric ferricyanide mixture (after Unna).

Amino-acids			KMnO₄	Iron mixture
Asparagine	...	...	–	
Alanine	...	...	–	–
Phenylalanine	...	...		–
Leucine	...	...	+	+
Glutaminic acid		...	+	+
Glycokoll	..	...	+ +	–
Cystine...	...	...	+ +	
Tyrosine and Tryptophane			+ + +	+ + +

The amino-acids *par excellence* which give the Berlin blue reaction are tyrosine and tryptophane, and to prove that these plasma cells contained tyrosine we stained some sections in Millon's reagent and succeeded in getting the recognised reaction. Millon's reaction was, on the other hand, very feebly marked in the syphilitic bodies; therefore, the reducing substance of the syphilitic bodies is not dependent upon tyrosine for its property.

So far as amino-acids are concerned we can say that the syphilitic bodies contain none free. Feeling that the protein molecule existed as such, we first directed our attention to the albumoses and repeated all Unna's experiments.

(a) *The protoplasmic portion of the syphilitic organism.*

The arbitrary division of albumoses is, in our opinion, not quite justifiable, and to say that granoplasma is a very special albumose, as Unna attempts to do, can scarcely be correct, since the granoplasma of connective tissue cells behaves differently from that of plasma cells; the greatest difference is also to be noticed in the individual plasma cells themselves depending upon their stage of development and degeneration, and lastly that of organisms and protozoa is as different again, and all behave differently according to the method of fixation.

Unna states that granoplasma is a deuteroalbumose and not a primary albumose. Although it differs from the former owing to its greater insolubility and in this respect resembles an acroalbumose which belongs to the latter group, the assumption is made that granoplasma is a deuteroalbumose, which has probably been formed from an acroalbumose.

To make this very complicated part of our work as clear as possible, it may be said that the most soluble granoplasma is that met with in the connective tissue cells and groundwork, then comes the granoplasma of some plasma cells, then of other plasma cells, then of the embryonic lymphocytes and nucleoli, and finally of the syphilitic bodies; but here we must halt for a moment, as in the syphilitic bodies we are dealing with two distinct proteins, one rather granular, which stains lighter with pyronin, the other

highly refractile, which stains deeply with pyronin; the former is the groundwork or granoplasma of the cell and resembles ordinary granoplasma; the latter may cover the whole cell or only the nucleus and is extremely resistant to reagents, and therefore does not resemble ordinary granoplasma; this is the protein which we will call the pyroninophile substance.

As the granoplasma of the syphilitic bodies resembles ordinary grano-plasma, the protein of the syphilitic bodies will be referred to as the pyroninophile substance, since it is the chemistry of this substance that we wish to unravel.

Unless otherwise stated, the following experiments were undertaken with sections which had been fixed in 50 % alcohol and which were placed in the different reagents for 12 hours at room temperature and then stained with pyronin and methyl green.

1.   In distilled water granoplasma begins to dissolve, the action is very much quicker at 37°, but the syphilitic bodies remain unaltered.   If kept in for several days, the avidity for pyronin disappears and the nucleic acid is left behind to stain with methyl green.   One may say that the protein of the syphilitic bodies is insoluble in water; this rules out protoalbumose and deuteroalbumose.   If normal saline is substituted for distilled water, the action is much the same and the syphilitic bodies are still insoluble; by this test acroalbumose is excluded.

2.   30 % alcohol behaves like normal saline and dissolves a greater portion of the granoplasma, but has no action on the syphilitic bodies. In 60 %, 70 %, 80 %, 96 %, and absolute alcohol the granoplasma remains mostly intact, depending upon the concentration, as no granoplasma is soluble in absolute alcohol.   In no percentage of alcohol are the parasites dissolved; this excludes at once peptone and protoalbumose, also deuteroalbumose α, which is soluble in 70 % alcohol and all the other deuteroalbumoses which are soluble in 80 % alcohol and upwards, except thiodeuteroalbumose and glycodeuteroalbumose β II, both of which are insoluble in absolute alcohol.

3.   In a 10 % solution of metaphosphoric acid granoplasma and the syphilitic bodies are insoluble, owing no doubt to the precipitation of all proteins by the acid; this is likewise the case with 1 % phosphomolybdic acid, alone, or with 1 % hydrochloric, 1 % phosphotungstic acid, picric acid, and weak solutions of the mineral acids.   These tests rule out protoalbumose, which is soluble in picric acid and dilute mineral acids, and Kühne's heteroalbumose which is also soluble in dilute mineral acids.   It is very difficult to work with the above acids owing to the fact that they all prevent staining with methyl green, and everything stains a diffuse red with pyronin.

4. Granoplasma and the proteins of the syphilitic bodies are insoluble in all strengths of acetic acid, and the reason why the nuclei stain green, giving the first impression that the pyroninophile substance over or in it has been dissolved, is only due to the marked precipitating action of acetic acid on nucleic acid.

5. Granoplasma is very soluble in a 1 or 2 % solution of boric acid but insoluble in a 5 % solution; the syphilitic bodies, on the other hand, retain their affinity for pyronin. Such a pretty instructive picture is obtained by leaving a section in 1 % boric acid for 12–20 hours at ordinary temperature and then staining in the usual way with pyronin and methyl green, that more than a passing mention is desirable. The granoplasma of all the cells has dissolved, the nucleoli have vanished, those embryo lymphocytes which stain red and might be confounded with certain phases of the syphilitic organism now all stain a brilliant green, and the only bodies which stand out a brilliant red colour are the syphilitic parasites. So in this very simple method we have as fine a differential stain as the Ziehl Niellsen for tubercle bacilli.

6. In 1 % potassium ferrocyanide not only does the ordinary granoplasma disappear, but the protein of the syphilitic bodies does also, with the result that only the nuclei stain with methyl green. If acetic acid is added to the potassium ferrocyanide the granoplasma and protein of the syphilitic bodies remain unaltered and stain in the ordinary way.

7. In a 2 % solution of copper sulphate the granoplasma has gone, nuclei remain, nucleoli have disappeared, also the granoplasma of the aminoplasma cells, the groundwork protoplasm of the syphilitic female bodies has vanished, but the pyroninophile substance over the nucleus remains intact and stains with pyronin, and most of the spore cysts stain red.

8. In 1 % caustic potash nuclei and all have dissolved.

9. In mercuric chloride and alcohol there is no change.

10. The syphilitic bodies remain unchanged after treatment with a 1–10 % solution of lead acetate; nucleoli are likewise not dissolved in this reagent.

From these experiments it is clear that the pyroninophile protein of the syphilitic parasites is not ordinary granoplasma, it is not an albumin, albumose or peptone; this leaves us with only globulin. So when the insolubility of the protein under question is considered, we think we are justified in saying that it is a globulin, or, as we shall see later, a globulin complex. If the sections have been fixed in absolute alcohol which prevents the extraction of salts and acts as a very powerful coagulant, many of the

above-mentioned substances fail to make any alteration, boric acid, for instance, being innocuous, and the syphilitic protein does not dissolve in potassium ferrocyanide. To produce the same results sections must be left in the reagents for several days.

50 % alcohol can extract electrolytes from the cells, hence they become less negatively or positively charged and the charge may be still further diminished by reagents, and as electrolytes are essential for the staining of fixed specimens, their complete abstraction will result in the pyronin not staining. Hence, it may be quite wrong to say that this or that protein dissolves in this or that solution, as it may be only its electrolytes which are removed; therefore, as previously stated, the arbitrary division of the cell proteins is not justifiable.

Absolute alcohol extracts few, if any, of the electrolytes, hence staining is not interfered with. As the syphilitic protein when fixed in 50 % alcohol resists reagents so remarkably, it can from what has just been said be assumed that the salts or electrolytes are firmly bound up with the protein. This would not be the case unless the protein was itself also bound up in a highly organised and stable complex, so we have the first clue in this simple observation that the pyroninophile substance of the syphilitic parasite is a protein (globulin) complex.

(b) The nuclear portion of the syphilitic organism.

After treatment with acetic acid before staining with pyronin and methyl green, or employing Ehrlich's triacid mixture, in order to get the nuclei of the syphilitic bodies to stain with methyl green, on careful examination marked differences can be discerned between the parasitic nuclei and those of other cells. In the former the methyl green stain gives a purer green colour, the stain is more brilliant and it is evenly distributed throughout the nucleus, or in other words, is homogeneous. If small lymphocytes be now examined, or the nuclei of plasma cells, and contrasted with the above, it will be noticed that the green is darker and has a mixture of blue or black; it is duller and moreover distributed into dots and strands, which are the chromatin bodies and filaments. If attention is now paid to the dividing cells and the embryo lymphocytes it will be noticed that the green resembles that met with in the syphilitic bodies and that the stain is again homogeneous. The only phase of the syphilitic organism which at all resembles in colour the lymphocytes or nuclei of the plasma cells is the spore cyst, or rather the sporozoites which

the cyst contains. This very simple observation is yet another very important argument in favour of McDonagh's view, since the nuclei of the developing syphilitic bodies resemble those of the developing lymphocytes and the dividing plasma cells, while the sporozoites which have no further need to develop and are in the resting stage resemble the mature lymphocytes and resting plasma cells. Degenerated nuclei behave quite differently; the division into chromatic filaments becomes more marked, the methyl green stains them bluish, then not all and at this stage the slender chromatic filaments stain red with the pyronin, or a diffuse red stain of the remaining protein may result; therefore, the syphilitic bodies are not degenerated nuclei.

The chemical substance of the nucleus is a compound of a protein and nucleic acid and is therefore called a nucleoprotein. This nucleoprotein on hydrolysis breaks down into protein and nuclein; the nuclein into protein and nucleic acid; the nucleic acid into purine bases, viz. guanine, adenine, xanthine and hypoxanthine, pyrimidine bases, viz. thymine, cytosine and uracil, pentoses and phosphoric acid.

Methyl green only stains nuclein and nucleic acid, whilst the protein stains with pyronin; therefore the reason why degenerated nuclei stain in some cases with pyronin, is that the nucleic acid has become further split up, while the protein remains behind.

(c)   Action of reagents on nuclei.

We next tried a series of experiments by leaving sections which had been fixed in 50 % alcohol for 20-24 hours at room temperature in several reagents to see if we could get any different reactions with the nucleic acid of the syphilitic bodies, and that of ordinary cells. The sections were stained with pyronin and methyl green, and every experiment which was undertaken was also repeated with sections of the soft roe of a herring (*Clupea harengus*).

1.   Leaving a section of roe for 20 hours in a concentrated solution of ammonia results in a partial disappearance of the nucleic acid, but the cells still stain with methyl green and retain their form; the chief difference from the normal is the appearance of a diffuse mass of protein which stains red with pyronin and is no doubt a mixture of histone and protamine.

In the syphilitic section the nucleoprotein has also been broken up; in some nuclei the nucleic acid has disappeared altogether, in other nuclei there are masses of it left as the red field is dotted here and there with some blue masses (methyl green). The granoplasma of the cells has dissolved. The

nuclei of the syphilitic bodies have partly gone and the granoplasma has completely disappeared and also the pyroninophile substance.

Nucleoli have mostly gone, and the aminoplasma cells completely. The best maintained of the syphilitic bodies are the sporozoites in the spore cysts which still stain quite intensely with methyl green and are, on the whole, even less damaged than the nuclei in the herring's roe. Therefore, the sporozoites are not only extremely rich in nucleic acid but also extraordinarily resistant to chemical reagents.

2. After leaving sections of roe in saturated sodium chloride solution all the nucleic acid disappears and all that is seen is a diffuse mass of histone which stains well with pyronin.

In the syphilitic sections the granoplasma of the cells is well preserved, if anything the pyronin staining of the protoplasm of the plasma cells is increased. The nuclei on the other hand are very much altered; they stain homogeneously a slate-grey colour; the chromatic bodies and filaments are not to be seen, but the nucleic acid is less disturbed than in that of the fishes' roe. The nucleus of the syphilitic parasite does not stain with methyl green, not because the nucleic acid is dissolved but because the brilliant refractile pyroninophile substance has been precipitated and therefore has had its properties intensified.

The nucleoli are well preserved and the aminoplasma cells are intact.

3. A section of roe which has been treated with a 1 in 3 solution of magnesium sulphate has lost all its nucleic acid, no cell outline is even discernible and all that remains is the precipitated histone, which stains especially brilliantly with pyronin.

In the syphilitic sections the granoplasma has partly dissolved, the nuclei stand out, stain a brilliant green and are intensely refractile, looking like pieces of green glass. The great difference between the nuclei found in the roe and in the syphilitic section can possibly be explained by the fact that in the latter the nucleic acid has not been extracted before the precipitation of the histone, with the result that the nucleoprotein is maintained, as is the case in sections which have remained in potassium ferrocyanide. All nucleoli have vanished. The nuclei of the syphilitic phases stain a brilliant green and are much better preserved than the nuclei of other cells; all the pyroninophile substance has completely disappeared.

4. The only difference noticed in a section of roe which has been in a 0·1 % solution of calcium chloride is that a trace of histone has been abstracted from the nuclei.

In the syphilitic sections the staining is not quite as good as under

ordinary circumstances. The pyroninophile substance is not destroyed, but the sporoblasts and sporozoites show a greater affinity for methyl green than usual; therefore a trace of the body is soluble in calcium chloride. These sections show up some points which we have frequently observed in other sections, and which have more than once raised the question as to whether we were dealing with spore cysts or the degenerated nuclei of plasma cells.

In some spore cysts which stain with methyl green, bright red bodies are to be seen resembling nucleoli. There may be one or more, varying in size, and frequently the largest red mass lies on and looks as if it was part of the biggest spore body. In this latter observation lies the solution. This large spore body is a part of a sporoblast which has to divide still further to form sporozoites and, as mentioned above, the oldest sporozoites have generally lost their pyroninophile constituent, which would not be the case with undeveloped sporozoites. The pyroninophile substance is no doubt separated off and breaks up into fragments in the groundwork of the spore cyst.

When the nucleus of a plasma cell degenerates it does not break up into a mass of circular bodies, but into a ring of bodies, some of which are circular and others oval; and there is nothing in the centre except a few strands which stain red with pyronin.

5. In 2·5 % sodium chloride all sections show the points brought out by the preceding reagent in a slightly more pronounced degree.

6. 20 % ammonium sulphate solution has the effect of so breaking down the nucleoprotein of the nuclei found in a section of roe that no distinct nuclei are seen, only a diffuse red purple colouration of the nucleic acid being left upon a deeply red diffuse groundwork of histone.

In syphilitic sections destruction is not nearly so marked, so far as the *toute ensemble* is concerned; the pyronin stain seems to have been increased probably owing to some abstraction of histone from the nuclei, as the staining capacity of the nuclei is very much weakened. The pyroninophile substance is maintained.

If specimens fixed with absolute alcohol are used, quite different results are again obtained. Nuclei are not affected by concentrated ammonia, but the pyroninophile substance dissolves. In 20 % NaCl nucleoprotein has been split up and part of the nucleic acid dissolved, but the pyroninophile substance is unaltered. If the syphilitic bodies are closely examined, a clear white halo surrounds the nucleus and tiny areas of white are to be seen in the nucleus. The refractility of the protoplasm of the plasma cells is markedly diminished while that of the syphilitic bodies appears to be increased, which has the effect of strongly differentiating them from the other cells.

7. In 0·6 % lithium carbonate the granoplasma of cells has mostly disappeared, the nuclei stain homogeneously and of a bluish colour; nucleoli are well maintained, and the syphilitic bodies remain practically unaltered. So here again is a fine differential method.

(d) Action of sera on cells.

We thought it possible that something might be learnt by treating sections with different sera, so the following experiments were undertaken.

Several cc. of blood were withdrawn from a non-syphilitic, a case of early secondary syphilis, and a case of late tertiary syphilis. Great care was taken to have the serum absolutely free from haemoglobin, and when put into the watch glass, a covering of pure toluene was used to prevent any bacterial action. Unless the latter precaution is taken bacteria multiply in the sera and exert a pronounced hydrolytic action on the sections so that even after 20 hours the individual cells are only just discernible. All sera have the same action as normal saline. If specimens fixed with absolute alcohol are treated in the same way, there is no change and in neither case can any difference be detected between the action of the three sera.

Whether the pyroninophile protein of the syphilitic bodies is part and parcel of the nucleus or only its sheath is at first sight difficult to ascertain, but we incline to the latter view for the following reasons.

Morphologically it looks more like a cover, this being especially noticeable in potassium permanganate specimens which have been counterstained with methyl green, as the nucleus stains faintly and appears hazy and gives exactly the impression of being covered with a veil. It is soluble in potassium ferrocyanide and when sections are left for 12 hours in a 1 % solution the nuclei stain better than ever and appear clearer with methyl green, which would scarcely be the case if it was part of the nucleus. Moreover, it gives a faint Berlin blue reaction, which no nucleus is known to do. Further, by staining with dextrose-borax-methylene blue, during impregnation, there appears to be a marked differentiation of ecto- and endoplasm and even the spirochaetae are swollen. If it is not part of the nucleus, in what combination with protein does the nuclein and nucleic acid of the syphilitic bodies exist? That a protein does exist is quite clear, since the nuclei of the syphilitic parasites can be made to stain with acid dyes, viz. diazine green, which mixes very well with pyronin; and moreover, will stain well with pyronin when the nucleic acid has been separated off, when it is found to behave like ordinary granoplasma. In this respect the protein

radicle of the syphilitic nucleoprotein does not differ from that of ordinary cells.

The protein of nucleoprotein is always regarded as quite a special protein, and said to possess strong basic properties and to be extremely rich in hexone bases, viz. lysine, arginine and histidine.

Nucleoprotein is a complex in which the properties of the protein can be very materially altered by the prosthetic group, since we are by no means aware what all the other constituents are and therefore cannot tell when they have been removed; we can never be sure that we are dealing with the protein only.

We have already shown that the division of the proteins of the cell protoplasm is purely arbitrary and the separation of the nucleoproteins is likewise no doubt artificial, since when the protein of the nucleoprotein is separated out it gives the same micro-chemical tests and behaves in the same way to stains as does the cell granoplasma; therefore the syphilitic nucleus does not differ in gross details from the nuclei of its host's cells.

For lysine and arginine we have as yet been unable to find a specific microchemical test, as immersing sections first into a freshly prepared solution of diazobenzene-sulphonic acid and then washing with a 1% solution of NaOH, did not give the characteristic pink colour which is seen in the microchemical test for arginine. Reversing the solutions by using the alkali first made no difference. Whichever method was employed a beautiful stable orange colour, which stained both the protoplasm and the nuclei, resulted.

The well-known test for histidine, namely, bromine water and acetic acid, also gave negative results, both before and after hydrolysing with a 0.5% solution of hydrochloric acid. Oddly enough a section which had been treated with bromine water and acetic acid quite failed to give the orange colour with diazobenzene-sulphonic acid and sodium hydroxide, which looks as if either the histone or the hexone bases had formed a halogen compound, which of course they are well known to do, and the bromo-histidine compound, for instance, does not give the characteristic reactions of the base.

Failing to get the typical hexone reactions we did not expect nor did we succeed in getting a positive imidazole reaction by treating sections with ammonia and silver nitrate. We also tried, but once more with negative results, to get a positive biuret reaction which is obtained with histone; the failure was due to the destruction of the tissues by the strong soda solution, which is unfortunately necessary for the reaction.

All nuclei are stated to contain phosphorus, which is intimately bound up with the nucleic acid radicle of the nucleoprotein and disappears when the

nucleic acid is hydrolysed, and as it is the nucleic acid radicle which shows the affinity for methyl green it is possible that this acid plays a part in the selective action of nuclei for methyl green. The nuclei of the syphilitic parasites, when the pyroninophile membrane covering them is removed or prevented from staining, stain not only brilliantly with methyl green but also show a greater resistance to hydrolytic agents than do the nuclei of ordinary cells; hence it might be expected that the parasitic nuclei were especially rich in phosphorus.

To see whether this surmise was correct, the following experiments were undertaken :

Phosphorus. Both fresh sections and specimens, fixed with absolute alcohol, the latter giving quite as good results as the former, were placed in a mixture of molybdic acid, ammonia and nitric acid, and kept therein at 37° for 10 minutes to 48 hours. One is supposed to regard as inorganic phosphorus that which makes its appearance in the first 10 minutes, but when sections are examined so early only negative results are obtained. After 24 hours good staining effects can be obtained, but the staining is sharper if the sections are left in the mixture for even another day.

The sections are washed well in distilled water and are then placed in a 2 % solution of phenylhydrazine hydrochloride, taken direct through alcohol, and mounted in balsam.

The presence of phosphorus is indicated by a green colour, and when the sections are examined it is found that both the protoplasm and the nuclei of nearly all cells are stained. The staining is deepest in the syphilitic bodies and then in the plasma cells; there appears to be no great difference in the staining properties of the nucleus compared with the cell protoplasm, which indicates that the phosphorus in a cell is not restricted to the nucleus.

As nuclei also contain iron the following tests were undertaken :

Iron. A. Inorganic.
B. Organic.

(A) *Inorganic.* Specimens fixed with absolute alcohol were after removal of wax transferred direct from absolute alcohol into a freshly prepared solution of equal parts of 0·5 % hydrochloric acid and 1 % potassium ferrocyanide, and allowed to remain therein for one hour. By this means the cells did not give the Berlin blue reaction, nor did they even stain green.

Instead of the hydrochloric acid potassium ferrocyanide mixture, a 0·5 % haematoxylin solution was employed, which also failed to prove the presence of inorganic iron.

(B) *Organic.* Sections prepared as above were placed in a mixture

of sulphuric acid (4 vols.) and absolute alcohol (100 vols.) and left therein at 37° for 24–48 hours.

After 24 hours the sections were washed in absolute alcohol and some were transferred for half an hour into the hydrochloric acid potassium ferrocyanide mixture, whilst others were stained in 0·5 % aqueous solution of haematoxylin. From both solutions the sections were taken through alcohol, etc. and mounted in balsam. When the former were examined it was seen that the protoplasm of the cells remained unstained while the nuclei stained green, the arrangement of the chromatin remaining unaltered. Other nuclei stained a light Berlin blue and the colour was homogeneous; examined for action on polarised light they were found to react slightly, while one of the other cells showed a trace of reaction. These cells were no doubt the syphilitic parasites; the nuclei of which contained more organic iron than those of the leucocytes and connective tissue cells.

The same difference in degree of staining was also noticeable in the haematoxylin specimens.

Summary. The protoplasm of the syphilitic bodies is strongly pyronino-phile, which proves it to possess reducing properties. The reducing action is not so strong as that of the aminoplasma cells and therefore is not due to an amino-acid; and moreover it prefers basic to acid dyes which still further distinguishes it.

The protoplasm is very resistant to reagents and in all respects resembles a globulin. The nucleus in its behaviour to dyes and reagents most closely simulates the nuclei of dividing cells.

Hence, neither can the syphilitic bodies be taken for cell degenerations nor nuclear degenerations.

FURTHER POINTS CONCERNING THE REDUCING ACTION OF CELLS, WITH SPECIAL REFERENCE TO THEIR PHYSICAL CHARACTERS.

None of the proteins we have mentioned have sufficient reducing power to form Berlin blue from the ferric ferricyanide reagent; therefore, the reducing action of some of the syphilitic bodies, red blood corpuscles, etc., must be due to a carbohydrate, a fat or a lipoid.

Carbohydrates. Our endeavours to find a carbohydrate in the syphilitic bodies failed and the tests used are only of interest from the negative information derived therefrom.

Keeping sections for some time in a hot solution of Fehling's reagent gave no yellow precipitate. Leaving sections for 48 hours at 37° in a solution

of 0·5 % potassium hydroxide in 90 % alcohol and then immersing them into a 2·5 % solution of dimethylparaminobenzaldehyde in 1 % HCl gave no carmine reaction, which indicates the absence of glucosamine.

Treating sections with a 15 % alcoholic solution of α-naphthol and then examining them in sulphuric acid for the furfurol reaction, owing to the destruction of the tissue, gave no information.

The principal organic tests and group reactions either necessitate the use of strong acids or alkalies ; with the former the tissue is as often as not dissolved *in toto* ; with the latter it is almost certain to leave the cover slip, swell and become practically unmanageable. Ammonia is the least offender in this way, but unfortunately it cannot as a rule take the place of the potassium or sodium hydroxides.

We tried also leaving fresh and fixed sections in a saturated solution of copper acetate at 40° for 24 hours and then washing them in a strong solution of sodium carbonate. A general reduction of the copper occurred in the fresh sections, but the individual cells could not be studied. In the fixed films the only cells which reduced were those of the rete malpighii. Therefore, although we can say that the tissue cells do contain sugar, we cannot differentiate them individually.

We therefore resorted to a physical test.

By the use of Nicol's prisms it is seen that the syphilitic cells when properly focussed appear as bright stars against a black background. The phenomenon is better marked in specimens fixed with absolute alcohol than when 50 % alcohol has been used for the same purpose, and can be beautifully demonstrated in sections stained with pyronin and methyl green. It is most marked over the nuclear area, is greater in zygotes than in female gametocytes, is very well marked in the trophozoite and male gametocyte and much less evident in sporoblasts and sporozoites. In short, it is greatest where the pyronin staining is deepest ; therefore, it is the pyroninophile substance that is concerned. The only cell constituents which exhibit this property are cholesterol, sugar, and lipoids.

Cholesterol gives a crystalline, and not a star-like appearance as seen in the syphilitic parasites, and moreover cholesterol is not increased in the serum of syphilitic patients. Sugar can be excluded, because if it were present in sufficient quantities to show the phenomenon, it would give the micro-chemical tests ; moreover sugar is soluble in water which the pyroninophile substance is not. Further, the active substance of the parasitic cells must therefore be lecithin, or rather its fatty acid constituent. To bring still greater evidence to bear on this assumption we compared the colloidal particles in a case of

pseudochylous fluid with dextrose and in a case without dextrose, with the result that the former were more active than the latter, the activity of which was comparable in degree with that of the syphilitic cells. The pseudochylous fluid with dextrose gave a marked Berlin blue reaction, which was not the case with the other. The fluid containing no dextrose had a reducing action equal to that of the syphilitic cells; therefore it appears justifiable to state that the syphilitic bodies contain no dextrose.

Leaving sections for hours and even days in ether, absolute alcohol, and absolute alcohol and ether mixed, the cells still retain this property and therefore the lecithin cannot exist alone. The pyroninophile substance contains protein, and as a lecithin-protein complex is known to exist and the protein is a globulin which we have also shown the pyroninophile substance to be, we can assume that this substance is lecithin-globulin.

Fatty acids. Now what evidence have we for assuming that it contains a fatty acid? We stained sections with iodine and with Nile blue sulphate, according to the method of Lorrain Smith. The syphilitic bodies stained with iodine and also very deeply with a saturated solution of Nile blue sulphate. Red blood corpuscles also stained with iodine and all nuclei and nucleoli stained with Nile blue sulphate. The only granoplasma that stained with Nile blue sulphate was that of the syphilitic bodies and some of the plasma cells. The inclusion of a fatty acid in the lecithin-globulin complex is still further supported by the invariable occurrence of a saturated fatty acid (stearic acid) in the lecithin-globulin complex of pseudochylous fluids, and the visibility of the fluid between crossed Nicol prisms is due to the fatty acid and not to the lecithin itself.

The reducing action of red blood corpuscles, syphilitic bodies, protoplasm of some plasma cells and nucleoli is in all probability due to the lecithin-globulin complex and the change that takes place in impregnated female cells is an increase of this body which, owing to its reducing action, prevents the cell from staining with methyl green, and, owing to its acid action, prevents the cell from showing a marked affinity for negatively charged dyes, as carbol fuchsin, etc. Since the complex is not so marked in unimpregnated female cells and the protein is less acid, it will stain with carbol fuchsin in the carbol fuchsin-carbol iodine green method. Owing to the staining reactions, the fatty acid must be a saturated one, and as neither the syphilitic bodies nor the colloidal particles of pseudochylous fluid stain with osmic acid or Sudan III, the fatty acid is clearly not oleic acid.

As the syphilitic parasite was shown to contain lecithin we thought it necessary to repeat the more important work which had been done in

connection with the lecithin in nerve tissue, so the following tests were carried out.

1. The tissue was fixed in Müller's fluid for 10 days, frozen and sections cut. The sections were transferred into 1 % osmic acid for 24 hours at 37° and then placed in the following mixture :—

Pyrogallic acid	...	...	parts 15
Sod. sulphite	...	...	,, 125
Sod. nitrate ...	...	...	,, 70
Water	...	...	,, 300

and differentiated in 0·1 % potassium permanganate which reoxidises the osmium which has not combined. The brown colour of the permanganate can be removed if desired with 1 % oxalic acid.

Considering how insoluble the syphilitic lecithin appeared to be in alcohol, we tried alcohol fixed specimens and paraffin sections, but instead of floating the cut sections out on to warm water, we employed a hot 7 % solution of potassium bichromate and obtained quite as good results. The syphilitic bodies had a greater reducing action upon the osmium than other cells, staining a deep olive green, the only other structures which resembled them were nucleoli and the aminoplasma cells.

2. Tissues were fixed for four days in 10 % formalin and were then placed for the same length of time in Weigert's chrome alum copper acetate mixture in the incubator at 37°. Some sections were cut in the frozen state, others taken through paraffin. The cut sections were put into sulphuric acid alcohol (1 : 500 H_2SO_4 in 50 % alcohol), and then stained for 10 minutes in 1 % osmic acid ; they were then well washed and treated with 5 % pyrogallic acid solution, differentiated in 0·1 % potassium permanganate taken through sulphurous acid and the fresh sections were mounted in liquid paraffin ; while the paraffin sections went through the usual stages to balsam. The results tallied with those obtained from No. 1.

3. Alcohol fixed specimens, the paraffin sections of which had been floated out on to potassium bichromate, were stained for 24 hours at room temperature in Weigert's haematoxylin.

The syphilitic bodies stain intensely and in this respect resemble the nucleoli. The chromatin of nuclei also stains well, but the colour is not so fast.

4. Paraffin sections of tissue which had been floated out on potassium bichromate were stained for 24 hours at 37° in Kultschitzky's haematoxylin, washed and decolourised in a 0·25 % solution of sodium carbonate until the sections retained a light blue colour. The sections were then washed,

transferred to a 0·1 % solution of potassium permanganate, then into sulphurous acid, washed in a 0·1 % solution of lithium carbonate, taken through alcohol and mounted in balsam.

The sections gave the same results as were obtained from method No. 3.

All sections were compared with and controlled by sections from a case of neurofibromatosis, with the result that the reducing action of the syphilitic bodies was practically equal to that of the medullated nerve fibres, and the capacity for staining with haematoxylin resembled that of the axis cylinders, the deep colour of which is no doubt due to the presence of a lecithin-protein complex.

Summary.   The reducing action of the pyroninophile substance of the syphilitic bodies is not due to cholesterol or a carbohydrate.   Although it is active optically, it is not so markedly so as the two substances just mentioned.

The activity is due to a fatty acid and resembles that of the particles met with in the fluid from a case of pseudochylous ascites.   The particles of such a fluid consist of lecithin-globulin with a saturated fatty acid (stearic acid) in its radicle.   This physical phenomenon is a most useful means of picking out the syphilitic bodies in a section.

OXIDISING ENZYMES.

Our next efforts were to ascertain whether the presence of an oxydase or a peroxydase could be detected in the syphilitic bodies, since some observers have stated that the bodies which have been described as parasitic are really mast cells.   For this purpose we fixed some tissue in formalin embedded in gelatin, and cut sections after freezing the material with carbon dioxide.

In testing for oxydases, the following methods were employed :—

1.   Sections were placed in a 1 % alcoholic solution of benzidine, but failed to stain, proving that no oxydase could be demonstrated; therefore, the syphilitic bodies do not harbour a tyrosinase, a ferment which at first sight might be expected to be present, owing to the increased pigmentation which is somewhat characteristic of syphilitic lesions.

2.   Sections were placed in a 1 % alkaline solution of α-naphthol (prepared by heating 1 grm. α-naphthol in 100 cc. dist. water and then adding just sufficient KOH to bring the α-naphthol into solution) for a few minutes and then transferred to a 1 % sol. of tetramethylparaphenylene-diamine hydrochloride (violamine) washed in distilled water and mounted in glycerol or liquid paraffin.   This method also failed to show the presence of an oxydase.

The following methods used for demonstrating the presence of a per-oxydase met with more success :—

1.   Sections were placed in a mixture of equal parts of a 1 % alcoholic solution of benzidine and 3 % hydrogen peroxide.

Such sections can be taken through alcohol, etc. and mounted in balsam, or of course mounted in the ordinary way.   A very strong peroxydase reaction was found in the red blood corpuscles and mast cell granules.   The syphilitic bodies remained unstained.

It is owing to the presence of peroxydases that the mast cells stain orange with pyronin.

2.   Equally good results can be obtained by leaving sections in a mixture of paraphenylenediamine tartrate (ursoltartrate) and H_2O_2.

The presence of a peroxide in tissues cannot be demonstrated, as no blue colour results on leaving sections in a mixture of benzidine and extract of horseradish.   The above affords sufficient evidence against the view that the syphilitic bodies are mast cells.

PHYSICAL AND CHEMICAL PROPERTIES OF THE LECITHIN-GLOBULIN COMPLEX.

The lecithin-globulin complex when in solution gives rise to a definite opalescence, and exists as a colloidal suspension which can be removed by filtration through a Chamberland candle.   It appears microscopically in the form of very fine refractile granules which do not stain with osmic acid, or Sudan III.   These fine granules no doubt owe their refractility to the associated lipoid lecithin.   The particles exist in an alkaline medium and possess a negative charge, and in consequence we find that the lecithin-globulin complex is readily precipitated by kations, particularly the divalent kations, Mg, Ba, and Ca.

The lecithin-globulin compound is readily precipitated by acetic acid even in the cold, also by alcohol : half saturation or full saturation with ammonium sulphate, and removal of the salts by dialysis results in separation of the lecithin-globulin complex.   Treatment of the complex with ether has no effect and previous addition of alkali such as caustic potash does not appreciably alter the solubility of this body.   In all respects this lecithin-globulin behaves exactly like the pseudo-globulin fraction isolated from serum. One third saturation with $(NH_4)_2SO_4$ precipitates the euglobulin fraction from serum and this fraction is soluble in a 0·6 % solution of sodium chloride,

whereas the pseudo-globulin remains insoluble. The pseudo-globulin fraction of the serum thus behaves in every way like the lecithin-globulin compound. The solubility of the globulin present in serum or in the body cells will therefore depend upon the amount of lipoid in association with the globulin, and the former will influence the optical properties, the electrical charge on the colloidal particles in suspension, and also the relationship of globulin to electrolytes. In connection with these observations, it may be noted how important both constituents of the complex are in the maintenance of life. During starvation, for example, the blood contains a larger amount of globulin, and after excessive bleeding the first constituent of the blood to return to its normal amount is the globulin fraction.

With regard to the nature of the lipoid present in association with the globulin, it is usually found to be lecithin. This lecithin is generally of the type described as a monoaminophosphatide, yielding choline on hydrolysis, and fatty acids of the stearyl group. The lecithin is insoluble in water, and is so firmly united to the globulin as to remain undissolved when treated with ether. The production of lecithin is probably determined by the processes of degeneration of cellular material, which takes place in diseases in which effusions may result, viz. tuberculous infections, malignant disease, and syphilis. In the production of milky effusions it seems highly probable that the destruction of lecithin-containing cell elements takes place in the blood itself, and that the lecithin so formed diffuses through the peritoneal membrane into the serous cavities. This would explain the sudden changes from a clear effusion to a milky one noted by some observers, or the reverse condition where a milky is later replaced by a clear transparent fluid.

The resistance to putrefaction exhibited by all fluids containing this complex is very striking in view of the fact that lecithin readily undergoes auto-oxidation when free. The complex seems to confer increased stability upon both constituents, but at the same time the lecithin is capable of fully exerting all its special functions, particularly its influence on the neutralisation of toxines and bacterial growth. The chemical configuration of the lecithin molecule possibly accounts for this property, since it contains a large number of hydroxyl groups capable of uniting with such bodies as ferments, proteins, sugar, and other lipoids. The power lecithin possesses of resisting putrefaction suggests a possible function of this body in the production of immunity.

From what has been stated it will be noticed how close is the resemblance between the pyroninophile substance of the syphilitic parasite and the colloidal particles of the pseudochylous fluid, both substances being no doubt

identical and the relationship becomes the closer when the staining properties of both are considered, and for brevity's sake we need only state that both the colloidal particles and the syphilitic parasites are Gram negative.

REFERENCES.

McDonagh, J. E. R. (1913, 1), *Proc. Roy. Soc. Med.* (*Path. Sect.*), **6**, 85.
—— (1913, 2), *Dermatolog. Wochensch.* **56**, 413.
Unna, P. G. (1913), *Biochemie der Haut* (Verlag von Gustav Fischer).

NOTE:

CHICK & MARTIN. *The Precipitation of egg-albumin by ammonium sulphate.* This Journal, VII, 1913, p. 380. In Table VIII, p. 392, 4th column, the values given for the percentage by weight in the Pressed Precipitate of $(NH_4)_2SO_4$ and water respectively should be interchanged and the Table should read thus:

Exp.	Composition, % by weight of	In pressed precipitate
I	$(NH_4)_2SO_4$	6·39
	H_2O	29·83
II	$(NH_4)_2SO_4$	8·47
	H_2O	29·91
III	$(NH_4)_2SO_4$	6·39
	H_2O	22·04

LI. ENZYME-ACTION, FACTS AND THEORY.

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The researches of the last few years on the kinetics of enzyme action have brought more confusion than clearness into this field. Even about the action of the best studied enzyme, invertase, there is contradiction and uncertainty; it seems as yet not quite established if, or under what conditions, the hydrolysis of cane-sugar by invertase follows the simple law of mass-action.

As to the nature of enzyme action there is a general inclination to suppose, that some chemical combination of enzyme and substrate plays a leading part in the process and that the general properties of the colloids, to which enzymes seem to belong, will supply sufficient explanation of their remarkable activities.

The aim of this paper is to clear up some of the contradictory statements in the literature about the kinetics of the most simple enzyme actions, and to show at the same time that there is another theory, which is more in accordance with the peculiarly characteristic behaviour of enzymes.

The hydrolysis of cane-sugar by invertase has been chosen by many investigators as the best process on which to study the velocity of enzyme action in relation with the different factors, which are here of importance. Both enzyme and hydrolyte are readily accessible, the action is simple, starts with the well-known substance saccharose and leads to the also fairly well-known substances glucose and levulose.

Chemically speaking, this hydrolysis is a monomolecular reaction. It was therefore a confirmation of what chemists expected, when O'Sullivan and Tompson [1890] found in an elaborate investigation, that the inversion of saccharose by invertase from yeast proceeded in accordance with the law of mass-action, i.e. if y represents the inverted fraction at a time t, $k = \frac{1}{t} \log \frac{1}{1-y}$ was nearly a constant.

Duclaux [1898] however, without doubting the correctness of the experimental results of O'Sullivan and Tompson, pointed out, that there was still a remarkable difference between the action of acids and invertase (and of enzymes generally).

In differently concentrated solutions of saccharose the same amount of acid gives the same velocity-constant k, thus for instance a 5, 10 or 15 % saccharose solution will be inverted by a 1 % acid solution in the same time to the same extent, e.g. half way. But for invertase Duclaux had found as early as 1883, that the same quantity of enzyme, acting in different concentrations of cane-sugar, inverted in the same time not the same fractions but the same absolute amounts.

Henri [1901] then took up again the experimental study of invertase and found, that not only was there the difference between the actions of enzymes and acids, established by Duclaux, but that k did not even remain constant during one and the same action of invertase on cane-sugar; on the contrary steadily increased.

Now it cannot be denied, that Henri's experiments were open to criticism owing to his not taking sufficiently into account the mutarotation of the freshly formed glucose in his polarimetric estimations.

It was therefore a valuable contribution on the experimental side of the subject, when A. Brown [1902] also investigated the kinetics of the invertase-action and found the same regular increase of the constant $k = \frac{1}{t} \log \frac{1}{1-y}$ for sufficiently concentrated saccharose solutions as Henri. On p. 375 of his article Brown says: " Conditions of experiment similar to those used by C. O'Sullivan and Tompson were employed, but the invertase used was prepared in a different manner from the enzyme with which these authors experimented."

To this last point we will return further on.

In order to remove all doubt, possibly also expressed by others as to the correctness of Brown's estimations, I wrote to him on this subject and was assured, that he " was well aware of O'Sullivan's experiences with regard to mutarotation and took exactly the same precautions to avoid errors in connection with it, which O'Sullivan adopted."

As I myself [1904] found the same deviation from the simple law of mass-action as Brown, without using a polariscope at all, but estimating the hydrolysis of cane-sugar by the accurate gravimetric method of Kjeldahl with Fehling's solution, Hudson's [1908] criticism of his predecessors (excepting Henri) must be rejected. How it was, that Hudson could again find validity

of the simple formula of mass-action in his experiments with invertase was explained by the work of Sörensen [1909].

In his elaborate article on: "The measurement and importance of the concentration of hydrion in enzyme-action" this author shows, that low concentrations of hydrion in the invertase-solution give the well-known increasing k. Going beyond this concentration one can work with an invertase-solution, where the reaction velocity follows the formula

$$k = \frac{1}{t} \log \frac{1}{1-y},$$

and in still more distinctly acid solutions k diminishes even more and more rapidly during the reaction. That this irregularity in the reaction-velocity is due to a gradually decaying of the enzyme by the action of the hydrion was perhaps not sufficiently emphasised in this article, though it is clearly expressed on p. 146 for the series with the highest hydrion concentration, where the reaction did not even come to a finish.

Indeed it follows from Sörensen's observations and deductions, that it is dangerous to work with enzymes at their optimum concentration of hydrion as well as at the optimum temperature of their activity. Both factors in that condition give splendidly rapid enzyme action, but at the same time are gradually destroying the enzyme itself.

It is obvious, that, where the quantity of the active catalyst is diminishing during a reaction, the velocity of this reaction will diminish at the same rate and may thus seem to fulfil the simple formula of mass-action. With low hydrion concentration, that is without adding acid or using extracts that have become acid by deterioration, one always gets for invertase (provided the saccharose solution is sufficiently concentrated) the same increase in the constant k, calculated as $k = \frac{1}{t} \log \frac{1}{1-y}$, or a real constant k, when it is calculated according to the empirical formula of Henri or to the theoretical formula, which I developed in 1904.

Sörensen drew attention to the fact, that O'Sullivan and Tompson brought their solutions by the addition of sulphuric acid to about the maximum of activity of the invertase, while Hudson worked with solutions containing acetic acid. Now we can add to this, that Brown "employed in (his) experiments an extract of invertase, prepared from dried yeast by digestion with water," and "without the complicating addition of sulphuric acid." In my own experiments I used an extract of fresh yeast, mixed with kieselguhr and dried rapidly at a low temperature. No acid at all was used.

When examining the results of O'Sullivan and Tompson as closely as these authors themselves did, one sees clearly, that the acidity they employed had not reduced the real velocities quite to an extent sufficient for the law of simple mass-action to be perfectly imitated. On p. 846 they say: "But if we look more closely at the diagram, we see that the deviations from the theoretical line, though small in themselves, are constant in the three experiments; in fact they correspond in a very striking manner." And a little further: "These figures all show a continuous increase in activity up to a certain point, about 80 per cent. of inversion, and after that a large and increasing decrease." That is exactly what the gradual destruction of the invertase must produce.

Sörensen goes however too far, when he generalises this influence of hydrion concentration to suggest a sufficient explanation for the marked falling off of the constant $k = \frac{1}{t} \log \frac{1}{1-y}$ in the experiments of Armstrong with lactase. My own experiments as well as the theory of enzyme action, which I proposed some years ago, lead to a different explanation and to a real reaction-constant in this case also.

As the application of this theory may now be extended in some points beyond what was published before, it may be of interest to survey once more a part of this much entangled field of enzyme actions with the aid of this theory.

Any hypothesis of enzyme action must be founded on the principal characteristics of this kind of catalysis.

It seems more and more established that these are:

1.   That the same quantities of enzyme, brought into differently, but sufficiently, concentrated solutions of substrate, produce in the beginning the same action in the same time.

2.   That catalysis by enzymes has a marked specific character[1].

Starting from these general facts, I ventured the hypothesis [1904] that enzyme action spreads like radiation from an enzyme particle as centre, and that an enzyme particle contains the same molecule, which is liberated or acted upon by this enzyme, in some active state.

What kind of radiation this may be cannot yet be decided. It is however very remarkable, that an imitation of specific enzyme actions has been produced by Rosenthaler [1908] by electromagnetic oscillations. By placing

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[1] This specificity is known to enzyme workers to be probably not so absolute as is often taken for granted. According to the following theory a limited specificity must also be looked for.

his substrate solutions inside a solenoid, through which a frequently interrupted direct current or an alternating current was passed, a diastase-like action on starch was observed for a frequency of 440–480 per second, and a hydrolysis of proteins for one of 320–360.

In order to explain the principal facts it is necessary to make the natural assumption, that this radiation may be absorbed by the substrate itself, by the substances liberated from it by the enzyme action, or by any other foreign substance added to the solution. The possible absorption by the water itself must be practically the same in every case and can therefore be left out of account.

It is evident that the sphere, within which this radiation-like effect around an enzyme particle may be expected to be active, must be very small; otherwise enzyme actions would be instantaneous.

In the beginning the spheres of action in differently, but sufficiently concentrated solutions of substrate will be inversely proportional to that concentration; therefore in the first time-unit the same quantity of enzyme will produce the same amount of effect in these solutions, for practically all radiation is absorbed by the substrate only.

In more dilute solutions not all the radiation will reach a substrate particle before it is too much weakened by spreading. This explains the fact that in moderately dilute solutions the constancy of the amount of initial action has disappeared. In sufficiently dilute solutions of substrate we shall practically obtain the condition, that on the average no two particles of substrate are on the same radius of the radiation sphere, so that all these particles will be acted upon at the same time and the amount of action will be directly proportional to the concentration of substrate; a fact observed by myself [1904] as well as by many other investigators.

The advantage of a reasonable working hypothesis proves here again to be its giving an indication, where to look for regularities and where these are not to be expected.

This hypothesis can also be quantitatively developed and leads, as I have shown, to a theoretical formula for the reaction-velocity in sufficiently concentrated solutions of substrate. We shall call the initial concentrations of the substrate a, the same after a time t of reaction x (all expressed for instance in grammes per 100 c.c.), and the amount of action in the first time-unit by a given quantity of enzyme in 100 c.c. of these sufficiently concentrated solutions m. According to theory as well as to experiment m is independent of a.

If further, n is the ratio between the absorption power for the enzyme

radiation of the reaction-products and that of the original substrate, the general differential equation for the rate of reaction is:

$$- dx = m \frac{x}{x + n \, (a - x)} \, dt.$$

As however catalysis by enzymes is generally recognised to be as a matter of fact a balanced action, this formula for the rate of reaction can only be applicable, where there is evidence that the reverse action, the synthesis, does not come into play. It will be shown further on, that this happens to be the case as a rule in the experiments with two of the best studied and relatively simple enzymes, invertase and lactase.

Bearing in mind this restriction, we obtain by substitution of $\frac{a - x}{a} = y$ and integration, the general formula for the rate of enzyme action in sufficiently concentrated solutions of substrate:

$$\log \frac{1}{1-y} + \frac{1-n}{n} \, 0{\cdot}434 y = \frac{m}{na} \, 0{\cdot}434 t$$

expressed in decimal logarithms.

The two constants in these experiments can be determined in different ways.

They may be calculated from the observed action at two different time-intervals, or m can be directly found by estimating the change during the first time-unit and n from the same estimation in similar conditions, but in a solution, in which had also been dissolved a known amount b per 100 c.c. of the reaction-products.

In the first estimation the initial velocity will be

$$\left(\frac{dy}{dt} \right)_{y=0} = \frac{m}{a},$$

in the second, from

$$- \frac{dx}{dt} = m \, \frac{x}{x + n \, (a - x) + nb} \, dt,$$

we get

$$\left(\frac{dy}{dt} \right)_{y=0} = \frac{m}{a} \, \frac{1}{1 \div n \frac{b}{a}}.$$

Evidently the value of n will depend only on the nature of enzyme and substrate, and must therefore be the same in the experiments of different observers.

For invertase from ordinary yeast the estimations of all the investigators, who avoided the errors mentioned in the first part of this article, agree with the value $n = \frac{1}{2}$, and I found the same also by direct estimation of initial velocities.

As $(a - x)$ grammes saccharose give by inversion $\frac{360}{342}$ grammes invert-sugar, the rate of inversion of cane-sugar in sufficiently concentrated solutions by unaltered invertase is thus generally represented by the equation:

$$\log \frac{1}{1-y} + 0.393y = kt.$$

The enzyme lactase is not as readily accessible as invertase, therefore its action is not so often studied. Ordinary brewers- or distillers-yeast is inactive in regard to milk-sugar. It is necessary to employ the special yeast, which is known to be the principal active agent of "kephir."

In order to extract the enzyme lactase as fresh and as unaltered as possible from the living cell, which produces it, I prepared a sufficient quantity of a pure culture of "Saccharomyces kephir" and worked only with the fresh extract from this yeast, which had been mixed with kieselguhr and dried in vacuum at a low temperature [1906].

In estimating the velocity of inversion of an 8 % milk-sugar solution at 30° by this lactase, I found a series of figures, to which it will now be shown that the general equation:

$$\log \frac{1}{1-y} + \frac{1-n}{n} 0.434y = \frac{m}{na} 0.434t$$

can also be applied with the same success.

Only, as explained above, the value of n may be expected to be different from that for invertase.

Direct observations of the very large retarding influence of glucose and of galactose showed that n is here much greater than in the case of invertase.

Calculation from two estimations of the hydrolysis of lactose at different time-intervals gives about $n = 6$. Thus for lactose the velocity of inversion is represented by

$$\log \frac{1}{1-y} - 0.362y = kt.$$

For 8 % milk-sugar at 30° I found, estimating the hydrolysis again by the gravimetric Fehling-Kjeldahl method:

t (minutes)	y	$k = \frac{1}{t}\left(\log \frac{1}{1-y} - 0.362y\right)$
5	0.23	0.0061
10	0.38	0.0070
15	0.47	0.0070
20	0.57	0.0080
25	0.61	0.0075
40	0.72	0.0073
66	0.82	0.0068
90	0.90	0.0075

Armstrong [1904], though also emphasising the importance of working rapidly and using only fresh extracts, did not it seems give care enough to these precautions. Kephir-grains, an extract of which he used, are a mixture of kephir-yeast cells with lactic acid bacteria and other residues, all together a mixture of somewhat unknown history and origin. The extract will contain some acid and in fact was not very active, compared with that obtainable from fresh pure lactose yeast. The experiments lasted several hours and even days, so that it was necessary to add toluene. The risk of decaying of the enzyme during these long experiments at 35°–37° was therefore not sufficiently excluded.

Still the first series of his investigations (p. 506) calculated with our lactose equation, gives

t (hours)	y	$k = \dfrac{1}{t} \log \dfrac{1}{1-y}$	$k = \dfrac{1}{t}\left(\log \dfrac{1}{1-y} - 0.362y \right)$
1	0·221	0·1085	0·0285
2	0·312	0·0812	0·0247
3	0·384	0·0713	0·0244
4	0·458	0·0665	0·0250
5	0·515	0·0629	0·0256
6	0·566	0·0604	0·0263
10	0·69	0·0509	0·0259
17	0·842	0·0471	0·0290
23	0·924	0·0461	0·033
29	0·953	0·0457	0·034
38	0·98	0·0447	0·035

The last of these estimations are less trustworthy, owing to exposure of enzyme and of milk-sugar to the combined action of the slight acidity and the temperature of 35°.

The other series of Armstrong's article lasted still longer, the inversions were far from finished at the end, and proceeded only very slowly during the last 24 hours. Evidently the enzyme was here to a large extent destroyed.

There are further regularities in the working of these relatively simple enzymes, invertase and lactase, which only become apparent when the investigator takes sufficient care to work only with enzymes, extracted with caution directly from the fresh living cells.

As I have shown before, the experiments not only agree with the conclusion, drawn from our hypothesis, that generally all neutral substances as well as the inversion products must retard the enzyme action, but striking quantitative regularities also exist.

For *unaltered*[1] invertase from ordinary yeast the facts are these:

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[1] By drying the yeast at a high temperature or treatment with alcohol I got an invertase, the action of which was more retarded by levulose than by glucose.

If we represent the retarding power (or the absorption coefficient according to our conception) of glucose by $n = \frac{1}{2}$, then the retarding powers of levulose and of invert-sugar are found to be exactly $n = \frac{1}{2}$ also. For the retarding powers of galactose and mannose however the experiment gives the double value $n = 1$. Nearly all neutral substances give retardation of the same order as the sugars, e.g. dulcitol, mannitol, urea, salicin and other glucosides.

It is very remarkable, that milk-sugar alone does not appreciably retard the action of invertase. In connection with this may be mentioned the small absorption power that milk-sugar was also shown to have for lactase action, compared with that of all other sugars. The figure for the inversion couple from milk-sugar was found to be $n = 6$. Galactose retards twice as much as glucose, so for galactose the absorption power of lactose action is $n = 8$, that of glucose $n = 4$. For levulose, a hexose which is here not contained in the substrate, experiment gives also the sum of the absorption powers of the inversion products of milk-sugar, that is $n = 12$.

In connection with the recent work of Tanret [1905] and of Hudson [1908] I wish to draw attention here to another point about the action of invertase and maltase.

If enzymes, as seems now more and more probable, are able to decompose a substance as well as to actuate the reverse process, then invertase, maltase and lactase should produce an equilibrium between bihexose and hexoses. In the case of maltase Croft Hill [1898] seemed to have established some synthesis of maltose from glucose, but it was proved afterwards, that the bihexose formed with difficulty was, at least for the most part, another substance. As for invertase and lactase, the evidence of synthesis of cane-sugar and milk-sugar is even slighter.

The facts, that a fairly concentrated solution of cane-sugar is practically completely hydrolysable by means of invertase and that after all no appreciable enzymatic synthesis of maltose from glucose is observed, suggest the conclusion, that the hexoses, split off by these enzymes, undergo a secondary change, by which the reverse enzyme action is prohibited.

Through the investigations of the mutarotation of sugars, especially by Tanret and by Hudson, much clearness has been brought into our knowledge of the isomerism of the most important members of this group. It was proved by Tanret, that glucose, dissolved in water is at first totally in the α-form ($[\alpha]_D = 110°$) and then gradually changes into a mixture in equilibrium ($[\alpha]_D = 52\cdot5°$) of about two-thirds α-glucose and one-third β-glucose ($[\alpha]_D = 19°$).

As two-thirds of glucose in solution are finally in this α-modification, then, if the glucose, split off by enzyme action from cane-sugar or maltose should be originally in this same form, as is generally assumed, it is difficult to see why the reverse action of the enzyme should not produce a considerable amount of maltose.

Also the hydrolysis of cane-sugar by invertase should then not be expected to come to a finish.

The rate of mutarotation of this α-glucose, i.e. the velocity with which it comes to this equilibrium, was carefully measured by Hudson, who made a similar investigation of the mutarotation of levulose. Combining these investigations with his experimentally quite correct estimation of the velocity of inversion of cane-sugar by invertase, Hudson came to the conclusion, that it is really α-glucose, which is originally formed by enzyme action in this case.

As the mean value of 18 experiments he found however for the specific rotation of this "fresh" glucose 120·7°, while this figure for α-glucose is 110°. This difference is so considerable, that Hudson's work also seems to point to the non-identity of enzyme-made fresh glucose with α-glucose.

This result is in complete agreement with the experimental evidence I got in 1904 of an unstable form of glucose, which seems to be the primary product of enzyme action and which gradually is converted to ordinary glucose in solution, that is, as we know now, to a mixture of α-glucose and β-glucose, according to the new nomenclature of Tanret.

It was expected, if the inversion of maltose was accomplished by a very active maltase solution[1] rapidly enough for the velocity of inversion to be great, compared with the velocity of conversion of the freshly formed glucose, that after the real equilibrium point maltose⇌glucose had been reached, the rate of inversion would appear to be approximately constant.

The curve, which must result from these two simultaneous reactions, may be demonstrated with the aid of Fig. 1.

After the few minutes that are needed for the principal reaction to come to the real equilibrium point P, the relatively slow secondary change of unstable to stable glucose has practically not yet started. Being a mono-molecular reaction, this rate of change of glucose will be at any time proportional to the concentration of unchanged unstable glucose.

As soon however as the concentration of unstable glucose has been

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[1] In my article of 1904 I omitted to mention, that these very active maltase extracts of the dried yeast-kieselguhr were obtained by using water with a small amount of sodium hydroxide. Extraction with water only gave a maltase solution of smaller activity.

diminished by this change, the equilibrium between maltose and glucose is disturbed and part of the maltose is split again. As we have arranged the conditions so that this inversion of maltose is much quicker than the transformation of glucose, the concentration of unstable glucose and therefore the rate of its change will be maintained constant (in first approximation).
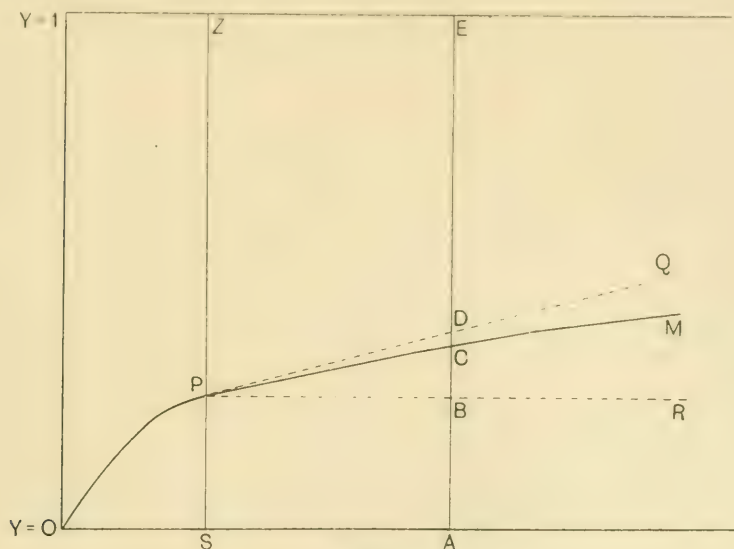
Fig. 1.

Thus in Fig. 1 we can represent, also in first approximation, the formation of stable glucose by the dotted straight line PQ, and the amount of unstable glucose by the horizontal dotted line PR. At any time, e.g. after A minutes, the equilibrium between maltose and unstable glucose will be immediately restored by the quick enzyme action. As however the concentration of maltose at the time A is somewhat smaller than that at the time S, the concentration of unstable glucose, required to balance by synthesis the hydrolysis of maltose, is also somewhat smaller than SP or AB.

The unstable glucose which has disappeared therefore need not be totally replaced by newly inverted maltose and the result will be, that at the time A this inversion will not have proceeded exactly to D, but to C. The estimations of the inversion of maltose will thus give a curve PM, slightly bending downwards from the straight line PQ.

Fig. 2, which illustrates the type of result obtained in my experiments of 1904, may serve also here to demonstrate the concordance with the theoretical type of Fig. 1.
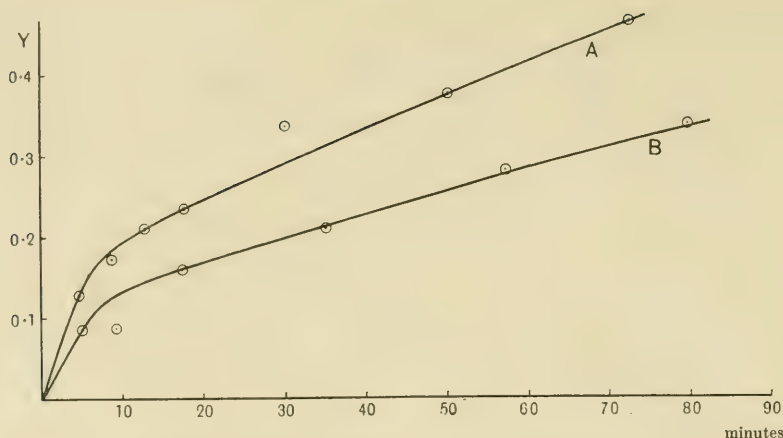
Fig. 2. A, 5 % maltose. B, 10 % maltose.

All the estimations of the hydrolysis of maltose were executed with Fehling's solution by the gravimetric method of Kjeldahl [1895] for mixtures of sugars.

These experiments with different concentrations of maltose showed further, that the positions of the equilibrium points, i.e. the points of flexure, where the curves become approximately straight lines, were connected together as these figures are in a balanced action between a monomolecular and a bimolecular reaction.

Following up the conception, that an enzyme particle extends its catalytic action in a sphere as regards both the reverse action and the hydrolysis of maltose, and that the size of this sphere in sufficiently concentrated solutions must be inversely proportional to the sum of the concentrations of maltose and glucose (the concentration of glucose multiplied by the absorption coefficient n), the complete formula for the velocity of action of maltase on maltose in conditions, where the secondary change of glucose is not yet perceptible, is

$$- dx = m \left\{ \frac{x - q\,(a-x)^2}{x + n\,(a-x)} \right\}\, dt.$$

After substituting again $\frac{a-x}{a} = y$, the equation, relating the equilibrium points (where $-\frac{dx}{dt} = 0$) with the initial maltose concentration, is:

$$1 - y - qay^2 = 0 \quad\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(E).$$

For a solution of 10 grammes maltose in 100 c.c. for instance, the experiment gave about $y = 0.15$, from which followed $q = 4$.

The other experiments with concentrations of 7, 5 (see Fig. 2), 3 and 1 g. for 100 c.c. agreed then with the figures, calculated from equation (E).

a	y
10	0·15
7	0·17
5	0·20
3	0·25
1	0·39

The synthesis of bihexoses in the living cell and the indications of synthesis in vitro, however slight they may be, suggest the conclusion, that the transformation of enzyme-made glucose to stable glucose is in fact also a balanced action, in which the equilibrium is far on the side of stable glucose.

In enzyme actions like this one, where the real equilibrium is so soon attained and the reverse action is thus vigorously hindering the hydrolysis, it is clear, that addition beforehand of the product of the reaction may retard more than that of any other compound of the same kind. The conversion of the unstable enzyme-made glucose is retarded considerably by the added glucose and with that the reverse action can assert itself.

For maltose I found a much larger retardation by glucose than by galactose and levulose.

Hence there is in fact no discrepancy between the peculiar retardation effects of foreign substances on the action of invertase and lactase on one side and the general rule, applied with success by ter Meulen [1905], on the other, that the enzymatic hydrolysis of a glucoside is most retarded by the same sugar, which is split off from the glucoside. The diagrams, representing the rates of hydrolysis of many of these glucosides, have the same character as that of maltose-inversion. Here also the reverse action would soon stop the further splitting of the glucoside, if the sugar, which is formed, were not liable to a secondary change, which change is effectively retarded by addition of a large amount of the stable form of the same sugar.

REFERENCES.

Armstrong (1904), *Proc. Roy. Soc.* **73**, 500.
Barendrecht (1904), *Zeitsch. physikal. Chem.* **49**, 456.
—— (1906), *Zeitsch. physikal. Chem.* **54**, 367.
Brown (1902), *J. Chem. Soc.* **81**, 373.
Croft Hill (1898), *J. Chem. Soc.* **73**, 634.
Duclaux (1898), *Ann. Inst. Past.* **12**, 96.
Henri (1901), *Compt. rend.* **133**, 891.
Hudson (1908), *J. Amer. Chem. Soc.* **30**, 1564.
Kjeldahl (1895), *Compt. rend. Carlsberg*, **4**, 329.
O'Sullivan and Tompson (1890), *J. Chem. Soc.* **57**, 865.
Rosenthaler (1908), *Sitzungsber. K. Preuss. Akad. Wiss.* 20.
Sörensen (1909), *Compt. rend. Carlsberg*, **8**, 120.
Tanret (1905), *Bull. Soc. Chim.* (3), **33**, 337.
ter Meulen (1905), *Rec. Trav. Chim.* **24**, 444.

LII. AN INVESTIGATION INTO THE PHYSICO-CHEMICAL MECHANISM OF HAEMOLYSIS BY SPECIFIC HAEMOLYSINS. (Preliminary Communication.)

By UPENDRA NATH BRAHMACHARI.

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The mechanism of haemolysis by specific haemolysins has not been much investigated from the physico-chemical standpoint. It is supposed that the haemolysin affects the permeability of the envelope. Baumgarten has noticed that the first stage of haemolysis, as produced by a biological poison, is the swelling of the corpuscles, just as occurs when the serum is made hypotonic. Bang [1910] considers that haemolysis by means of Cobra poison is due to change in the composition of the lipoid membrane of the erythrocyte rendering it more permeable to salts. Through this membrane the extracellular salts and water soak gradually, until in the end the corpuscle ruptures. The behaviour of the nucleated erythrocytes of amphibians towards specific haemolysins tends to shew that their permeability is alone affected during the process [Landau 1903].

So far as I am aware, no work has been done on the behaviour of erythrocytes loaded with amboceptor towards distilled water before the complement has been allowed to act upon them. The present paper gives the results of investigations on the determination of the resisting power of amboceptor-loaded human erythrocytes to haemolysis when under the influence of distilled water. The haemolytic antisera were obtained from fowls and rabbits that had been immunised by means of injections of washed human corpuscles.

It was originally expected that amboceptor-loaded corpuscles would be less resistant than normal ones in their behaviour towards distilled water. Contrary to what was expected, I noticed a remarkable increase of the resisting power of the erythrocytes.

The method adopted for determining the resisting power of the erythrocytes is that previously described by me [1909, 1911] for the determination

of the specific resistance of erythrocytes to haemolysis. A series of experiments was performed and these are described as follows:—

In the first series, the erythrocytes were suspended in 0·85% NaCl and allowed to haemolyse with two parts of distilled water. The mixture was allowed to stand for ten minutes and the dissolved haemoglobin was estimated in the manner previously described. This gave the resistance of the normal erythrocytes to haemolysis.

In the second series, the erythrocytes were mixed with two parts of a 10% dilution of complement-free antihuman fowl's haemolytic serum with 0·85% NaCl and kept in the incubator for one to three hours and subsequently treated in the same way as above with distilled water after having been thoroughly washed in 0·85% NaCl, and the resistance of the amboceptor-loaded erythrocytes determined in the same way as above.

In the third series, the erythrocytes were treated with complement-free antihuman rabbit's haemolytic serum and their resistance to haemolysis determined.

As a control test, a few experiments were made to determine the resistance of human erythrocytes after having been treated with normal fowl's serum in the same way as above.

In this way the resistance of the erythrocytes under different conditions was determined and the following tables worked out. The resistance of the erythrocytes is expressed in terms of the relative haemoglobin-value of the resistant erythrocytes which is the ratio between the amount of haemoglobin in the resistant corpuscles and the amount in the total suspension of erythrocytes used.

Tables shewing the resistance of erythrocytes to haemolysis under different conditions:—

TABLE I.

	Resistance of normal erythrocytes to haemolysis	Resistance of erythrocytes loaded with amboceptor obtained from fowl
1	0·345	0·500
2	0·379	0·456
3	0·242	0·407
4	0·333	0·480
5	0·346	0·452
6	0·269	0·531
7	0·333	0·466
8	0·350	0·444
9	0·301	0·421
10	0·288	0·459
11	0·288	0·368

TABLE II.

	Resistance of normal erythrocytes to haemolysis	Resistance of erythrocytes loaded with amboceptor obtained from rabbit
1	0·195	0·600
2	0·188	0·378
3	0·296	0·592
4	0·240	0·480
5	0·132	0·220

TABLE III.

	Resistance of normal erythrocytes to haemolysis	Resistance of erythrocytes treated with normal fowl's serum
1	0·288	0·243
2	0·288	0·298
3	0·282	0·259
4	0·240	0·282

It will be seen from the above tables that in every case the addition of the amboceptor increased the specific resistance of erythrocytes, while the addition of normal fowl's serum did not bring about any difference in their resisting power.

The problem that we are now to solve is how it is that the erythrocytes become more resistant when they become fixed to the amboceptor but lose all their resisting power as soon as the complement acts and what is the biological significance of this remarkable phenomenon. An explanation may be offered, which however is hypothetical. In the first process of the action of haemolysin during which the amboceptor combines with the erythrocytes, we may assume that this combination is purely of the nature of *adsorption* and not true chemical combination. In this process the dimensions of the pores between the molecules of the outer wall of the erythrocytes become small due to the amboceptor molecules filling the pores of the original erythrocytes. As diffusion depends, specially, upon the dimensions of the pores of the membrane through which it takes place, less haemoglobin passes out through the red corpuscles loaded with amboceptor than the unloaded ones when they are treated with distilled water. In the case of the colloidal complex of amboceptor and erythrocyte molecules, the bodies brought into close contact with each other do not react with one another in the chemical sense. Chemical reaction which, according to Bayliss [1912], is the third and last stage of the heterogeneous reactions of colloidal complexes, takes place between the molecules of the amboceptor and of the

erythrocytes only through the agency of the complement, which probably acts more or less like a ferment. When this takes place, there is a condensation of the molecules of the colloidal complex in each erythrocyte and as a result of this, the dimensions of the pores of the membranes of the red corpuscles increase to such an extent that haemoglobin diffuses out of the corpuscles and haemolysis results.

This theory will easily explain the inhibitory influence exerted by hypertonic saline solutions on the action of specific haemolysins as has been observed by Sutherland and McCay [1911] and others. Such saline solutions would tend to reduce the size of the erythrocytes by exosmosis of water from them and as a result of this the molecules of the erythrocytes come closer to each other and haemolysis is prevented. In other words the widening of the pores of the erythrocytes brought about by the action of the complement on the amboceptor-loaded erythrocytes is counteracted by the presence of the hypertonic saline.

To test the accuracy of the theory the following experiments were performed :—

A suspension of sheep's corpuscles in normal saline was mixed with amboceptor and complement and kept in the incubator. The process of haemolysis was stopped in from five to ten minutes before it was complete, the corpuscles quickly centrifuged and the supernatant fluid replaced by $N/2$ NaCl, after thoroughly washing the corpuscles several times with the same. A portion of the suspension of the corpuscles in $N/2$ NaCl was again centrifuged and the $N/2$ solution was pipetted off and in its place an excess of 0·85 % NaCl solution was substituted—a marked haemolysis resulted. Thus we have the following results :—

(1) Amboceptor-loaded corpuscles partially acted upon by complement, thoroughly washed in $N/2$ NaCl—no haemolysis.

(2) Amboceptor-loaded corpuscles partially acted upon by complement, thoroughly washed in $N/2$ NaCl and then the $N/2$ NaCl replaced by 0·85 % NaCl—distinct haemolysis.

In other words amboceptor-loaded corpuscles which have been partially acted upon by the complement are haemolysed when brought in contact with normal saline. The dimensions of the pores of such corpuscles when suspended in $N/2$ NaCl are much less than when suspended in normal saline, i.e. there is marked widening of these pores when they are suspended in normal saline and haemoglobin diffuses out. Evidently, therefore, the pores of the amboceptor-loaded corpuscles that have been acted upon partially by the complement are much wider than those of the normal ones. The

complement must have therefore brought about a condensation of the molecules of the amboceptor-loaded corpuscles, as explained before, thereby leading to widening of their pores to such an extent as to allow the haemoglobin molecules to pass out of them, even when they are suspended in normal saline.

An attempt was made to determine the volume of the corpuscles before and after combination with amboceptor. After rejecting different methods, the following method was devised for determining the size of the corpuscles. Human and sheep's corpuscles were used for the purpose.

The corpuscles were first suspended in 0·85 % NaCl and the suspension was sucked into two large-bore haematocrit tubes of exactly the same diameter and the corpuscles in both were centrifugalised at a high speed for about ten minutes, after which their volume was noted. The clear supernatant fluid from one of the tubes was now pipetted off and a dilution of haemolytic amboceptor in 0·85 % NaCl (one part of amboceptor and nine parts of 0·85 % NaCl) introduced in its place. The amboceptor was then thoroughly mixed with the corpuscles and the mixture allowed to stand for ten minutes. The other tube which was used as a control was then shaken up and the corpuscles again mixed with the supernatant fluid. The two tubes were again centrifugalised for ten minutes and the volume of the corpuscles noted. If the volume of the corpuscles in the control tube was found to be exactly equal to what was noted in it in the first experiment, then there was no error due to any change in the speed of the centrifuging tube. The volume of the amboceptor-loaded corpuscles was then noted. If the volume of the corpuscles in the control tube was different in the second experiment from what was noted in the first, then the volume of the amboceptor-loaded corpuscles was accordingly changed. In this way, absolutely accurate results were obtained.

Thus, if V be the volume of the corpuscles in the control tube after the first centrifuging and V' after the second, then $V - V'$ is the difference due to changes in the speed of the centrifuging machine.

If now v be the volume of the corpuscles before being loaded with amboceptor, and v' after being loaded with amboceptor, it is evident that $v - v'$ is not actually the change in the volume of the corpuscles. The exact change is, evidently,

$$(v - v') + \frac{v}{V}(V - V').$$

In this way, the exact change in the volume of the corpuscles due to combination with amboceptor was found and the following results obtained.

Human corpuscles.

Volume before action of amboceptor	Volume after action of amboceptor
(1) 2·75 cm.	(1) 2·75 cm.
(2) 2·75	(2) 2·75
(3) 2·50	(3) 2·50
(4) 1·50	(4) 1·50
(5) 1·45	(5) 1·50

Sheep's corpuscles.

Volume before action of amboceptor	Volume after action of amboceptor
(1) 3·30 cm.	(1) 3·30 cm.
(2) 5·38	(2) 5·20
(3) 4·50	(3) 4·20
(4) 6·23	(4) 6·22

It will be seen from the above tables that there was no reduction in the volume of the corpuscles after the action of the amboceptor and therefore the increase in their resistance to haemolysis was not due to any diminution in their volume after the action of the amboceptor.

I am deeply indebted to Lt.-Col. Sutherland, I.M.S., of the Calcutta Medical College, for kindly providing me with the haemolytic antisera and giving me every facility to work in his laboratory.

REFERENCES.

Bang (1910), *Biochem. Zeitsch.* **23**, 463.
Bayliss (1912), *Proc. Roy. Soc.* B, **84**, 81.
Brahmachari (1909), *Biochem. J.* **4**, 59, 280.
--   (1911), *Biochem. J.* **6**, 291. (See also *Studies in Haemolysis*, 2nd Ed.)
Landau (1903), *Ann. Inst. Past.* **17**, 52.
Sutherland and McCay (1911), *Biochem. J.* **5**, 1.

LIII. NOTES ON SOME FURTHER EXPERIMENTS ON THE CLOTTING OF CASEINOGEN SOLUTIONS.

By SAMUEL BARNETT SCHRYVER.

From the Research Institute of the Cancer Hospital.

Some months ago, I communicated to the Royal Society the results of a series of experiments on the clotting of caseinogen solutions [1913]. It was there shown that caseinogen, when freshly precipitated from diluted skimmed-milk under certain specified precautions, is an unstable substance which on treatment with water at 37°, or with 0·1 % acetic acid at room temperature, is readily converted into another product or series of products (metacaseinogens), which differ from the original substance in that they are very much less soluble in lime water, and consist therefore apparently of smaller aggregates of lower molecular weight. When a half-saturated solution of lime water is saturated with the natural caseinogen by rotating the mixture for several hours in a thermostat at 20°, an acid milky solution is obtained after centrifugalisation and filtration, which readily clots without the addition of a soluble calcium salt in the presence of rennin; it will also clot in the absence of rennin when gently warmed (to 25°) in the presence of soluble calcium salts when the latter exist in the mixture within certain definite limits of concentration. In the presence of milk-serum, peptones, amino-acids etc., a clot can be produced from the above-mentioned calcium caseinogenate solution only in the presence of both soluble calcium salts and rennin.

In the paper quoted it was shown, furthermore, that the casein of the clot produced from calcium caseinogenate by rennin alone is about half as soluble in lime water as the natural caseinogen, and appears to possess, therefore, approximately half the molecular weight, and corresponds in properties with the metacaseinogens produced from natural caseinogen by the action of water.

Similar conclusions as to the molecular weight of casein, and its relations to caseinogen have been arrived at recently by Lucius van Slyke and A. W. Bosworth [1913], who have attacked the problem from a quite different

standpoint, and by other experimental methods, and in a still more recent paper by Bosworth [1913] a method is described for the production of a clot from caseinogen solutions by rennin alone, which does not differ materially from the method described by me [1].

Bosworth also came to the same conclusion as I did, that the production of casein from caseinogen is not an ordinary proteoclastic reaction, although it may be the result of a hydrolytic cleavage. I have ventured to suggest that the two substances bear a relationship to one another similar to that of metaphosphates to pyrophosphates. Bosworth and I are also in accord with reference to the identity, or at any rate the approximate identity, of the empirical chemical compositions of caseinogen and casein.

Accurate stœchiometrical relationships between casein and caseinogen were not to be attained by the method of experiment adopted by me, owing to the existence of an adsorption equilibrium when lime water is treated with excess of various caseinogen and casein preparations, a point which has been the subject of numerous experiments, which are briefly mentioned in my previous paper [1913, p. 466].

The casein prepared from the clot differs in one important respect from metacaseinogen produced by the action of water at $37°$ or by 0.1% acetic acid at room temperature from caseinogen, for whereas the latter, on solution in excess of caustic alkali and reprecipitation from this solution with acetic acid under certain defined conditions is reconverted into a caseinogen of relatively high solubility in lime water, which readily yields clots on treatment either with calcium chloride or rennin, casein obtained by means of rennin cannot be converted into caseinogen under these conditions; on dissolving in alkali and reprecipitation, a product of only about half the solubility of caseinogen in half-saturated lime water is obtained. The solution in lime water furthermore does not clot on addition of rennin, and yields, on mixture with solutions of calcium salts within certain limits of concentration, an immediate precipitate. This precipitate is readily distinguishable from the clot produced under similar circumstances from caseinogenate solutions, which only forms when the temperature is raised above that of the temperature of solution.

Further experiments have been directed towards ascertaining the reason of this difference between the casein of the clot and the metacaseinogen

[1] Throughout my papers, I have consistently referred to the preparations which have not been clotted as caseinogen, whereas the preparations obtained from the clot are described as casein. In this respect, I have kept to the English method of nomenclature. Van Slyke and Bosworth adopt the method of nomenclature usual on the continent, describing the products as casein and paracasein, respectively.

produced by the action of water alone on caseinogen. These experiments are not yet complete, but I have been tempted to communicate them in their present form, owing to the fact that they have been subjected to a temporary interruption due to my change of laboratories.

CLOTTING OF CASEINOGEN SOLUTIONS BY MEANS OF PANCREATIN.

Investigations have been carried out with the object of comparing the process of clot production by means of other ferments. The results obtained with pancreatin indicate that its action can be readily distinguished from that of pepsin (or rennin). When half-saturated lime water is saturated with natural caseinogen, the solution thus obtained in the manner described [Schryver, 1913, p. 462] readily clots on the addition of small amounts of pepsin solution (1 %). *No soluble calcium salt need be added.* A clot under similar conditions cannot be produced by a pancreatin solution, although the latter will clot milk. A soluble calcium salt appears, therefore, to be necessary when pancreatin is employed for clot-production [compare Mellanby, 1912]. A clot was also prepared from an artificial milk by pancreatin in the following way: 5 c.c. of calcium caseinogenate solution were mixed with an equal bulk of milk-serum containing calcium chloride in the concentration of N/25. Under these conditions, as has already been shown, no clot is produced. If to this mixture are added a few drops of 1 % pancreatin solution, and the whole is incubated, a clot is produced similar in appearance to that produced by pepsin under like conditions.

The clot produced from artificial milk has the same properties as that produced from the natural milk, but differs in certain important respects from the clot produced by pepsin (or rennin) preparations.

The general method employed for examining the casein from the clots was as follows. The serum was poured off from the clot, which was then thrown on calico, and squeezed. It was then ground in a mortar with 0·1 % acetic acid, washed with water, dilute alcohol in increasingly graded strengths, absolute alcohol and ether, and then air-dried. The fine powder thus produced was then heated for 5 minutes with absolute alcohol to destroy the enzyme, the hot alcohol was filtered off by means of a Buchner funnel and the powder then washed with alcohol and ether, and again air-dried. The powder was then transferred to a mortar, ground with water into a paste, and N sodium hydroxide solution was added with continual grinding, until nearly all dissolved, and the solution was alkaline to phenolphthalein. It was then diluted and filtered through paper-pulp on to a Buchner funnel. Dilute

acetic acid was then carefully added with constant stirring until precipitation was complete, the supernatant liquid was rapidly poured off, and the precipitate washed by decantation or on a filter with ice-cold water, alcohol in graded strengths up to absolute alcohol, and finally with ether, and it was then air-dried.

Sometimes the above process was modified in various details, e.g. the clot after washing with water was first dissolved in alkali and reprecipitated, and the product after drying in the usual manner treated with hot alcohol. In all cases, however, a similar product was obtained. When the clots produced by pepsin and pancreatin were compared, the same procedure was always employed. The clots produced by pepsin alone from calcium caseinogenate solution, or by the combined action of the ferment and calcium chloride and ferment in the presence of milk-serum, yielded caseins with the same properties.

Now whereas the original caseinogen preparations had a solubility in half-saturated lime water of about 35[1], and after solution in alkalis and reprecipitation a solubility of about 30, the solubility of the casein preparation from the pepsin clot was about 14–17 and that of the pancreatin clot about 6–8. Unlike metacaseinogen, therefore, caseins are not converted into the more soluble products by solution in alkali and reprecipitation with acids, even when all precautions for preventing subsequent change have been taken. Furthermore, a saturated solution of caseinogen in half-saturated lime water is opaque like milk, whereas that of pepsin-casein is opalescent, and that of pancreatin-casein is generally water-clear. The two latter yield immediate precipitates and not clots on treatment with calcium chloride and do not clot on treatment with rennin. There appears, therefore, to be a difference in the molecular weights of the caseins produced by pepsin and pancreatin, both of which are considerably lower than that of caseinogen, that of pepsin-casein being about one-half and that of pancreatin-casein about one-quarter of that of caseinogen. Again, attention must be called to the fact that exact stœchiometrical relationships are not to be expected from the method of experiment employed.

The differences between metacaseinogens and caseins.

As already repeatedly stated, the metacaseinogens differ from the caseins in that the latter are not reconverted into caseinogen on solution in alkali

[1] I.e. 5 c.c. of the solution after Kjeldahlisation required 35 c.c. N/10 sulphuric acid to neutralise the ammonia produced (see former paper).

and reprecipitation with acids, whereas the former are. In spite of the large number of experiments which have been carried out, I have not so far succeeded in producing from a casein a solution which clots on treatment with rennin.

Investigations have, therefore, been carried out with the object of ascertaining the reason of the differences of behaviour between these substances, which in other respects have similar properties.

Up to the present, no entirely satisfactory proof as to the actual nature of the difference has been found, but certain facts have been recently discovered which afford some indication.

It seems not improbable that the ferment forms a combination with its substrate, from which it cannot afterwards be separated, and that the properties of the latter are then so altered that it can no longer form the big aggregates of the character of caseinogen.

Experiments were therefore carried out with the object of ascertaining the properties of the substance produced, when ferments were allowed to act on caseinogen at low and high temperatures, i.e., under conditions under which the proteoclastic action of the ferment is excluded.

Pepsin was allowed to act on calcium caseinogenate for 15 minutes at 0°, the conditions of experiment being otherwise the same as those employed in clot production. No clot formed under these conditions. The free caseinogen was then precipitated with ice-cold dilute acetic acid and a little sodium chloride[1], and rapidly washed several times with ice-cold water, graded strengths of alcohol, absolute alcohol and ether. These operations were carried out as rapidly as possible. The powder after air drying was then heated for 5 minutes with boiling alcohol, dissolved in excess of alkali, from the solution in which the casein(ogen) was obtained in the usual way. Although the product thus obtained possessed about the same solubility in half-saturated lime water as ordinary caseinogen which had been dissolved in alkali and reprecipitated, the solution obtained from caseinogen which had been treated with the pepsin differed from that got from normal caseinogen (treated in a similar way but without pepsin), in that the former did not clot in the presence of rennin, and yielded an immediate precipitate, and not a clot, when treated with calcium chloride solution of the requisite concentration.

The mere precipitation therefore, of caseinogen in the presence of a ferment under conditions which exclude enzyme action, causes appreciable alterations in its properties. When one considers the important part that surface

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[1] The precipitate does not readily separate in ice-cold solutions without this addition.

actions play in such processes, this result is not altogether surprising. The above result was obtained several times. A similar experiment was also carried out in which pancreatin was used instead of pepsin, the conditions being the same as those described above. The product in this case had a solubility of only about 8 in half-saturated lime water, and was identical in properties with the casein obtained from a pancreatin clot. It may be remarked here that pancreatin produces the clot in the highly acid solution.

A large number of experiments were also carried out with the object of ascertaining the action of the heated ferment on clot formation. The results obtained up till now are not, however, quite conclusive, and this is due to the varying properties of the different pepsin preparations. Rohonyi [1913] has recently succeeded in preparing definite compounds of proteins with proteoclastic ferments, and has shown that such compounds can be obtained when ferment solutions are employed, the proteoclastic activity of which has been destroyed by heat. If the conception is true that the caseins differ from metacaseinogens in the fact that the former are combinations with the ferments, then, in the light of Rohonyi's recent work, it is conceivable that caseins should be formed by the action of heated enzymes. In carrying out the experiments devised for testing this theory precisely the same difficulties have been encountered as those described by Rohonyi, due to the differences of characters of the various enzyme preparations. This investigator has shown that it is possible to prepare compounds of pepsin and proteins by the employment of solutions of the ferment which have been heated for 15 minutes at 100 *provided that no precipitate is formed during the heating.* Preparations having this property appear to be rare. I have myself not succeeded in obtaining such. I have, however, obtained a preparation which after heating to 80° for some time was still capable of actively clotting calcium caseinogenate solutions. Prolonged heating of the ferment solution in quartz tubes at this temperature produced a precipitate, and the solution gradually became inactive.

It is very probable that experiments of this nature will only succeed when a ferment preparation relatively free from extraneous proteins is obtained. Further work in this direction is contemplated.

The nature of "natural caseinogen."

L. van Slyke and Bosworth have called attention to the fact that various commercial preparations of caseinogen contain large quantities of acid calcium salt, due apparently to the precipitation of the product with insufficient quantities of acid. A criticism might therefore be directed against the "natural caseinogen" described by me, that such consisted of a mixture of free acid and acid calcium salt. I therefore subjected my preparation to examination with a view of ascertaining its nature, and comparing it with a calcium-free preparation prepared according to the method of van Slyke and Bosworth. The latter was found to have a low solubility in half-saturated calcium hydroxide, as it had stood some time in contact with hydrochloric acid during the process of filtration. After solution in alkali, and reprecipitation with acid, with the precautions already repeatedly described, it had a solubility of about 26, i.e., about the solubility of my own caseinogen preparations which have been "purified" by solution in alkali and reprecipitation by acid[1].

The solubilities of my "natural caseinogen" and of the caseinogen preparations prepared according to van Slyke and Bosworth (both the preparation directly precipitated with hydrochloric acid and that redissolved in alkali and reprecipitated by acetic acid with precautions for preventing change) in warm 10 % sodium chloride were determined. Van Slyke and Bosworth show that under these conditions the calcium salts readily dissolve, whereas the free acids do not. The preparations were mixed with 10 times their weight of the saline solution and maintained at 37° for about 1 hour with continual shaking. The mixtures were afterwards centrifuged, and the nitrogen in the clear filtered supernatant liquid was determined by Kjeldahl's process.

The saline extract from all preparations contained about the same small amount of nitrogen, viz. 2 or 3 mgrms. in 5 c.c. Only minute quantities of the preparations were soluble in saline, and my "natural caseinogen" is therefore a free acid and not a calcium salt. As a control, an acid calcium salt was prepared by precipitation of sodium caseinogenate solution by calcium chloride [Schryver, 1913, p. 469]. The precipitate was washed with dilute alcohol till chlorine free, and obtained dry after alcohol and ether washing in the usual way. On treating this with 10 times its weight of 10 % sodium chloride at 37°, it rapidly dissolved, leaving only a very small residue, and yielding a thick syrupy solution.

[1] Preparations thus "purified" have never as high a solubility in lime water as "natural caseinogen."

Conclusions.

1. Casein produced from a pepsin (or rennin) clot differs from meta-caseinogen, a product produced by the action of water at 37° on caseinogen, in that it cannot be converted by solution in alkali and reprecipitation into a more soluble product which dissolves in calcium hydroxide to yield clottable solutions. The statement made in my earlier paper that casein produced by pepsin is an aggregate of about half the size of that of caseinogen is confirmed.

2. The action of pancreatin in clot-production differs from that of pepsin, in that it produces clots only in the presence of soluble calcium salts. It produces these clots, however, under conditions under which calcium salts alone fail, e.g., in the presence of milk-serum. The casein from pancreatin clots also differs from that of pepsin clots. The former have only about half the solubility in half-saturated lime water of the latter. Such lime water solutions are usually water-clear.

3. Certain experiments are quoted, which indicate that the difference between metacaseinogens and caseins is due to the fact that the latter are combinations of the enzyme with the protein. The evidence produced both by myself and independently by van Slyke and Bosworth and by Bosworth alone, indicate that the action of the ferment is not an ordinary proteoclastic action. It is conceivable that if the enzyme contains both haptophoric and zymophoric groups, the former only take part in clot formation.

4. Evidence is brought to show that the "natural caseinogen," the preparation of which is described in my earlier paper, is not a calcium salt.

REFERENCES.

Bosworth (1913), *J. Biol. Chem.* **15**, 231.
Mellanby (1912), *J. Physiol.* **45**, 345.
Rohonyi (1913), *Biochem. Zeitsch.* **53**, 179.
Schryver (1913), *Proc. Roy. Soc.* B. **86**, 460.
Van Slyke, L. and Bosworth, A. W. (1913), *J. Biol. Chem.* **14**, 203, 207, 211, 227 and 231.

LIV. ON THE CHOLESTEROL CONTENT OF THE TISSUES OF CATS UNDER VARIOUS DIETETIC CONDITIONS AND DURING INANITION.

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Since the discovery of cholesterol by Conradi in 1775, and its analysis by Chevreul in 1815, it has been found to be very widely distributed in the animal, and in its isomeric forms in the vegetable, kingdom, and it is now generally recognised as an integral constituent of all cells in the animal body [Dorée, 1909]. The constant presence of such a substance clearly suggests its importance from a vital standpoint, and necessitates its recognition as a primary constituent of all protoplasm. Though a considerable amount of work has been done on the subject, however, we have as yet little definite knowledge of its physiological functions.

As a result of experiments on herbivorous animals published by one of the present writers and various colleagues and the researches of Pribram [1906], Kosumoto [1908, 1 and 2], Harley and Barratt [1903] the working hypothesis was put forward that in these animals cholesterol is a substance which is strictly conserved in the animal organism; that when the destruction of the red blood corpuscles and possibly other cells takes place in the liver, their cholesterol is excreted in the bile, and that the cholesterol of the bile is reabsorbed in the intestine along with the bile salts and finds its way into the blood stream to be used in cell anabolism [Dorée, Ellis, Fraser and Gardner, 1908–1912]. In the case of carnivorous animals there would not appear to be the same necessity for such economy, as cholesterol is a normal constituent of their food, whereas the food of herbivora contains no cholesterol, but instead the isomeric phytosterols, and these only in minute quantity. The absorption of the food cholesterol would, however, be limited by the reduction of cholesterol to coprosterol by the bacteria of the gut. In the

human subject cholesterol is excreted in the faeces entirely in the form of coprosterol under ordinary conditions [Bondzynski, 1896; Bondzynski and v. Humnicki, 1896], though it has been shown by Müller [1900] that a prolonged milk diet leads eventually to the excretion of cholesterol as such. In the case of dogs and cats the change into coprosterol is generally only partial, though under some conditions it may be complete, and takes place only on meat diets [Dorée and Gardner, 1908, 2]. Feeding experiments [Dorée and Gardner, 1908, 2; Kosumoto, 1908, 2; Ellis and Gardner, 1909, 2] on dogs and cats have shown that the total cholesterol found in the faeces, either as cholesterol or coprosterol can be more than accounted for by the cholesterol taken in with the food, provided that the animal remains in health and constant in weight. This is also probably the case in man [Ellis and Gardner, 1912, 3]. Feeding cats, however, on artificial diets free from cholesterol led to inconclusive results from the point of view of deciding whether in the case of carnivora the cholesterol of the bile is normally reabsorbed along with the bile salts in the intestine [Ellis and Gardner, 1909, 2]. Earlier experiments made by Dorée and Gardner [1909] to trace a connexion between the percentage of cholesterol in the blood of dogs and the cholesterol content of the food taken, also led to inconclusive results, partly owing to the defects in the methods then available for estimating cholesterol in the tissues. Recent improvements in the methods of estimating cholesterol—notably the digitonine process of Windaus [1910] —rendered it desirable to repeat and extend the experiments referred to. In the present paper we give an account of analyses of blood and the tissues of cats fed on diets containing different amounts of cholesterol and also of cats kept in a state of inanition.

It is much more difficult in the case of carnivorous animals to maintain constant conditions during experiment than in the case of herbivorous animals such as the rabbit. In the latter animals a natural standard diet, free from cholesterol and similar bodies, to which measured portions of cholesterol could be added was available; and as these animals are practically continuous feeders the bile flow and consequently the cholesterol content of the blood due to this source would remain practically constant. In the case of cats we had to make use of different foods, though the difference was not so marked as in the earlier experiments. Little is known concerning the influence of food on the secretion of the bile, but from experiments that have been made by various observers, there is good reason to suppose that the nature of the diet would not be without influence [vide Goodman, 1907]. Furthermore the cat and the dog are discontinuous feeders and the flow

of bile into the intestine would be intermittent. Under these circum-
stances the portion of the floating as distinguished from the constituent
cholesterol in the blood and the tissues due to the reabsorption of the
cholesterol of the bile, would not necessarily be strictly comparable in
different cases. What would be the limits of such variation, if any, we have
no data at present upon which to form an opinion, but a variation of the
kind suggested might wholly or partially mask any variation due to the
cholesterol absorbed from the food, which at best would not be great in
absolute magnitude. We did not therefore expect to obtain results comparable
in precision with those obtained in the case of herbivora, but we attempted
to render the conditions as comparable as possible by careful selection of
diets, and by always killing the animals during a period of active digestion.

Details of the diets used.

Diet A, cholesterol free. We had some difficulty in finding a cholesterol-
free diet which cats would take freely, but ultimately the following proved
suitable. The whites of four eggs were taken raw and mixed with an equal
weight of mashed boiled potatoes. The well-mixed material was flavoured
with a small quantity of Liebig's extract of meat, and heated until the white
of egg coagulated. The animals appeared to relish this diet, not the
slightest trouble being experienced in getting them to eat it.

Diet B. Diet A, with the addition to each daily ration of a weighed
amount of free cholesterol.

Diet C. Lean cooked beef.

Details of the animals used.

Diet A, cholesterol free.

Cat No. 1. The animal was fed for eight days and consumed 1003 grams
white of egg and 1003 g. potatoes, the total weight when cooked being 1932 g.
During this period its weight remained quite constant—2·7 kilos. It was
killed one hour after the last meal. The stomach was full, and all the organs
appeared normal.

Cat No. 2. This cat ate the same amount of food as No. 1, and during
the eight days of the diet period its weight was as follows :—2·67, 2·58, 2·6,
2·55, 2·5, 2·5 and 2·55 kilos. It was killed two hours after the last meal.
The stomach was full, and all the organs seemed normal.

Cat No. 3. This cat was pregnant, and during 11 days consumed

728 g. of egg white, 728 g. of potatoes, weighing when cooked 1396 g. It lost weight gradually during the diet period—3, 2·7, 2·7, 2·5, 2·4, 2·3 and 2·3 kilos. It was killed one hour after the last meal.

Cat No. 4. This animal ate in the course of 11 days 1138 g. egg white and 1210 g. of potatoes, weighing when cooked 2188 g. It remained approximately constant in weight—3·2, 3·2, 3·2, 3·1, 3·0, 3·1, 3·1, 3·1 kilos. It was killed *three* hours after the last meal. The stomach was half full, and the organs seemed normal.

Diet B.

Cat No. 5. During 11 days this cat consumed 693 g. of egg white and 693 g. of potatoes, weighing when cooked 1256 g., and 2·75 g. of cholesterol, i.e. about twice the amount it would have got from the same weight of meat. It weighed 2·3 kilos. at the beginning of the experiment and 2·2 kilos. at the end. It was killed one hour after the last meal, and all its organs were quite normal.

Cat No. 6. This cat consumed during 11 days 728 g. of egg white and 728 g. of potatoes, weighing when cooked 1303 g., and 2·75 g. of cholesterol. It weighed 2·4 kilos. at the beginning and gradually decreased in weight to 2·1 kilos. It was killed 1·5 hour after the last meal. Its organs seemed normal.

Diet C.

Cat No. 7. This animal was fed for ten days on lean cooked beef as free as possible from fat and consumed 1563 g. of meat weighing in the raw state 2165 g. This would correspond to an intake of about 1·7 g. of cholesterol. The weights taken from time to time were 3·37, 3·3, 3·4, 3·33, 3·42, 3·45 and finally 3·43 kilos. It was killed four hours after the last meal. The stomach was full and all the organs normal.

Cat No. 8. This cat consumed during 11 days 1910 g. of cooked lean beef, weighing in raw state 2433 g. This would correspond to an intake of about 1·9 g. of cholesterol during the period. The animal remained constant in weight—3·3 kilos. It was killed four hours after the last meal. The stomach was full and the organs normal.

ANIMALS IN A STATE OF INANITION.

In these experiments well-fed animals were kept without food for seven days, but were allowed as much water as they wished. At the end of this period they all appeared to be in good health. No faeces were passed.

Cat No. 9. The weights taken daily, except on Sunday, were 3·1, 2·9, 2·8, 2·6, 2·55 and 2·5 kilos. The loss of weight was therefore 19·4 %. After the animal was killed the stomach was found to be completely empty. There was a little fluid in the small intestine, and some faecal matter in the rectum.

Cat No. 10. The weights recorded were 3·1, 2·95, 2·8, 2·75, 2·7, 2·6 and 2·5 kilos., the loss in weight being as before 19·4 %. The stomach was quite empty; a small amount of fluid was found in the small intestine and some faeces in the rectum.

Cat No. 11. This animal similarly treated only lost 10 % of its body weight, the weights recorded from time to time being 2·8, 2·7, 2·6, 2·5 and finally 2·5 kilos. The stomach was empty, but there was some fluid in the intestine and faeces in the rectum. The weight of the contents was 92 g., and this was found to contain 0·24 g. of cholesterol.

Immediately the animals were killed, they were cut up and the various tissues submitted with the least possible delay to the treatment described below. The following were examined—blood, liver, muscle taken from various parts of the body, kidney, suprarenal glands, heart, brain, lung and contents of the gall-bladder.

METHOD OF EXTRACTION AND ESTIMATION OF CHOLESTEROL.

The tissues were finely minced and ground up with sand and enough plaster of Paris to make the whole mass fairly dry and friable. It was allowed to stand until quite dry, powdered and extracted in a Soxhlet's apparatus with ether for about 14 days. The ethereal extract was made up to known volume, and divided into two portions, or in some cases into suitable aliquot portions. In one portion the free cholesterol was estimated and in the other the total free and ester cholesterol after saponification in ethereal solution by means of sodium ethylate. The cholesterol was estimated by the modification of Windaus' digitonine process described by Fraser and Gardner [1910]. The ester cholesterol was thus determined by difference, and it was assumed that no hydrolysis of the esters took place in the time occupied in dissecting out the tissue, mincing up and drying with plaster of Paris. Though we think this assumption is probably justifiable in the case of most of the tissues, it is perhaps open to some question in the case of liver. This tissue is known to contain ferments, which hydrolyse cholesterol esters, but the hydrolysis does not appear to take place rapidly even under most favourable conditions. Thus a sample of liver which on treatment as

described above was found to contain 0·28 % of total cholesterol and 0·13 % of free cholesterol and therefore 0·15 % of ester cholesterol, was still found to contain 0·05 % of ester after incubating for four days at 37° in saline suspension in the presence of a little toluene, though another sample allowed to putrify in saline suspension at 37° yielded no ester. We endeavoured however to reduce any such error to a minimum by carrying out the operations as rapidly as possible.

DISCUSSION OF ANALYTICAL RESULTS.

Blood. The results of the analysis of the blood of the eleven animals are collected together in Table I.

TABLE I.

No. of cat	Diet	Weight of blood taken for analysis, in grams	Total free and combined cholesterol in g. per 100 g. of blood	Free cholesterol in g. per 100 g. of blood	Cholesterol in form of esters per 100 g. of blood
1	A (non chol.)	68·2	0·044	0·044	Nil
2	A	87·9	0·060	0·046	0·014
3	A	84·1	0·058	0·056	0·002
4	A	83·2	0·044	0·040	0·004
		Mean of 4 cats	**0·052**	**0·047**	**0·005**
5	B (cholest.)	74·2	0·102	0·098	0·004
6	B	59·8	0·178	0·049	0·129
		Mean of 2 cats	**0·140**	**0·074**	**0·066**
7	C (meat)	126·2	0·083	0·066	0·017
8	C	74·2	0·078	0·054	0·024
		Mean of 2 cats	**0·081**	**0·060**	**0·021**
9	Inanition	65·8	0·163	0·074	0·089
10	Inanition	76·5	0·196	0·069	0·127
11	Inanition	75·6	0·151	0·126	0·025
		Mean of 3 cats	**0·170**	**0·090**	**0·080**

On comparing the figures in column four of Table I it will be seen that the total cholesterol of the blood increases with the cholesterol taken in with the food. In the case of the animals in a state of inanition, which were living on their own tissue, the increase is very marked. This increase is in all cases chiefly due to an increased quantity of ester cholesterol, though the free cholesterol itself also increases. The high ester content is very marked in the case of animals in a state of inanition. This may very likely have

something to do with the transference of the fat from the depots during starvation, and in this connexion it may be noted that in the case of cats 9 and 10 which lost 19 % of their body weight their blood contains 0·089 and 0·127 % of ester cholesterol, while that of cat 11, which only lost 10 % of body weight, is 0·025. In the case of cats 2 and 3, both of which lost weight during the diet period, the total cholesterol of the blood is somewhat higher than in the case of cats 1 and 4 which had the same diet but remained constant in weight. The same thing will be noticed in the case of cat 6 compared with cat 5 on the same diet.

The variations observed are similar to those found in the case of rabbits fed on diets containing varying amounts of cholesterol, and the figures are of much the same order as will be seen from Table II in which only average values are given.

TABLE II.

Number of animals of which the figures are the average	Animal	Diet	Total free and combined cholesterol per cent.	Free cholesterol per cent.	Ester cholesterol per cent.
4	Cat	Cholesterol-free A ...	0·052	0·047	0·005
4	Rabbit[1]	Bran extracted by ether ...	0·058	0·044	0·014
2	Cat	B (A + cholesterol) ...	0·140	0·074	0·066
4	Rabbit[1]	Extracted bran + cholesterol	0·081	0·059	0·022
2	Cat	C (lean beef)	0·081	0·060	0·021
2	Rabbit[1]	Ordinary bran	0·082	0·069	0·013
2	Rabbit[1]	Cabbage leaves and stalk ...	0·068	0·063	0·005
3	Cat	Inanition	0·170	0·090	0·080
2	Rabbit[1]	Inanition	0·154	0·113	0·040

[1] [Ellis and Gardner, 1912, 2.]

Liver. In Table III we give the total cholesterol, free and combined, actually found in the livers of the animals, also the weights of cholesterol per cent. of liver and the weights of liver cholesterol per kilo. of body weight. On comparing the figures for animals fed on diet A (cholesterol free) it will be seen that both the total percentages of cholesterol in the liver and the total liver cholesterol per kilo. of body weight are fairly uniform, though not so constant as was found to be the case in rabbits fed on cholesterol-free diet. On comparing these figures with those of cats 5 and 6 which were fed for a period on the same diet, but with the addition of free cholesterol, a marked increase in the total cholesterol is obvious. This increase is partly due to free cholesterol, but mainly to ester cholesterol. On the other hand in the case of cats 7 and 8, fed on lean meat, i.e. a diet containing

TABLE III.

No. of cat	Diet	Wt. of animal when killed, in kilos.	Wt. of liver, in g.	Wt. of total cholesterol in liver, in g.	Wt. of free cholesterol in liver, in g.	Wt. of ester cholesterol in liver, in g.	Wt. of cholesterol per cent. of liver			Wt. of liver cholesterol per kilo. of body wt.		
							Total	Free	Ester	Total	Free	Ester
1	A	2·70	59·8	0·128	0·092	0·036	0·214	0·154	0·060	0·047	0·034	0·013
2	A	2·55	54·6	0·136	0·041	0·095	0·249	0·076	0·173	0·053	0·016	0·037
3	A	2·30	63·0	0·102	0·102	Nil	0·162	0·162	Nil	0·044	0·044	Nil
4	A	3·10	70·8	0·192	0·048	0·144	0·254	0·067	0·187	0·062	0·015	0·047
					Mean values		**0·220**	**0·115**	**0·105**	**0·052**	**0·027**	**0·025**
5	B	2·20	48·4	0·194	0·089	0·105	0·401	0·185	0·216	0·088	0·041	0·047
6	B	2·10	43·7	0·228	0·049	0·179	0·466	0·117	0·349	0·108	0·023	0·085
					Mean values		**0·434**	**0·151**	**0·283**	**0·098**	**0·032**	**0·066**
7	C	3·43	77·5	0·243	0·221	0·022	0·316	0·285	0·031	0·071	0·064	0·007
8	C	3·30	107·7	0·362	0·362	Nil	0·346	0·346	Nil	0·109	0·109	Nil
					Mean values		**0·331**	**0·316**	**0·015**	**0·090**	**0·087**	**0·003**
9	Inanition	2·50	50·3	0·345	0·063	0·282	0·550	0·125	0·425	0·138	0·025	0·113
10	Inanition	2·50	49·8	0·295	0·069	0·226	0·637	0·143	0·494	0·118	0·027	0·091
11	Inanition	2·50	48·2	0·245	0·059	0·186	0·509	0·122	0·387	0·098	0·024	0·074
					Mean values		**0·565**	**0·130**	**0·435**	**0·118**	**0·025**	**0·093**

a considerable proportion of cholesterol but no added carbohydrate, there is a similar increase in total cholesterol, but the increase is almost entirely free cholesterol, the ester being nil in one case and considerably below the average of diet A in the other. In the case of cats 9, 10 and 11, in which the animals were living on their own tissues, we find a similar storing up of the cholesterol in the liver. It is stored almost entirely in the form of esters and there is only a slight increase if any of free cholesterol. This may also have something to do with the transference of the fat from the depots to the liver during starvation. In the case of cat 11, which lost only 10 % of body weight, this storing up is not quite so great; in this cat the percentage in the blood was also somewhat lower than in the case of cats 9 and 10. Those changes are generally similar to those observed by Ellis and Gardner [1912, 1] in the case of rabbits during starvation. Assuming that the loss in weight of the cats during inanition is due to muscle tissue, then, if the cycle postulated for herbivora be true for carnivora, cats 9 and 10 both of which lost 600 g. of body weight should have accumulated each about 0·5 g. of cholesterol in the blood, liver and the contents of the intestine. Taking the average figures for diet A as representing the normal cholesterol content of the blood and liver under conditions in which body weight is kept constant, but no cholesterol is absorbed with the food, 0·42 g. of cholesterol was accumulated in the blood and liver of cat 9 and 0·41 in cat 10. The

contents of their intestines were not analysed, but in cat 11 about 0·2 g. was found.

Suprarenal Glands. This organ was only examined in five of the eleven cats, and the results are given in Table IV.

TABLE IV.

No. of cat	Diet	Weight of organ, in g.	Total cholesterol, free and combined, actually found	Free cholesterol actually found	% of total cholest.	% of free cholest.	% of ester cholest.
3	A	0·6569	0·0116	0·0060	1·77	0·91	0·86
4	A	0·5886	0·0155	0·0034	2·63	0·58	2·05
5	B	0·6310	0·0373	0·0373	5·92	5·92	Nil
6	B	0·4408	0·0228	0·0070	5·13	1·59	3·58
11	Inanition	0·5208	0·0048	0·0035	0·92	0·67	0·25

Here the addition of cholesterol to the diet produces a marked increase in the total cholesterol content of the organ, and the change appears to run parallel to that in the blood. In cat 5, for instance, the increase in the blood is all free cholesterol, the actual amount of ester cholesterol being slightly below the average of the cats on diet A. In the suprarenal the increase is also all free cholesterol, the ester being nil or very small. In cat 6 on the other hand, where the increase is almost entirely in ester cholesterol, the ester cholesterol of the blood is very high, while the free cholesterol is almost exactly equal to the average on diet A. In inanition however this parallelism no longer appears to hold, for while the cholesterol accumulates in the blood it decreases almost to the vanishing point in the suprarenals. More experiments will however be required before this interesting point can be settled. The inanition result appears, however, to be in agreement with the observations of Landau [1913]. In this connexion it may also be noted that Albrecht and Weltmann [1911] and Hueck [1911] have shown that in diseases such as Carcinoma, Tuberculosis, etc., both the free and ester cholesterin of the suprarenals sink and sometimes are absent altogether.

Muscle. The muscle analysed was a mixed sample cut from various parts of the body. The results are given in Table V.

TABLE V.

No. of cat	Diet	Percentage total cholesterol	Percentage free cholesterol	Percentage ester cholesterol
1	A	0·056	0·031	0·025
2	A	0·047	0·032	0·015
7	C	0·087	0·083	0·004
8	C	0·081	0·073	0·008
9	Inanition	0·059	0·053	0·006
10	Inanition	0·065	0·051	0·014

It will be noticed that on diet A, which contained no cholesterol, but was rich in carbohydrate, there is a marked increase in the ester cholesterol compared with the cats on meat diet C. The free cholesterol is however much lower. In the case of rabbits, Ellis and Gardner were unable to trace any influence of food cholesterol on the cholesterol content of muscle.

Heart. The results are given in Table VI.

TABLE VI.

No. of cat	Diet	Wt. of organ, in g.	Total cholest. per cent.	Free cholest. per cent.	Ester cholest. per cent.
1	A	7·9	0·080	0·073	0·007
2	A	8·8	0·068	0·068	Nil
7	C	16·3	0·073	0·073	Nil
8	C	12·5	0·072	0·063	0·009
9	Inanition	10·5	0·071	0·063	0·008
10	Inanition	9·4	0·091	0·039	0·052

The cholesterol of this organ is very constant, and nearly all present in the free state. The percentage is much the same as that given for heart muscle in the ox, 0·066 to 0·071 by Ellis and Gardner [1908].

Kidney. The results on this organ are given in Table VII.

TABLE VII.

No. of cat	Diet	Wt. of animal when killed, in kilos.	Wt. of kidney, in g.	Total cholesterol, in g.	Free cholesterol, in g.	Ester cholesterol, in g.	Wt. of cholesterol per cent. of kidney			Wt. of kidney cholesterol per kilo. of body wt.		
							Total	Free	Ester	Total	Free	Ester
1	A	2·7	21·6	0·064	0·064	Nil	0·298	0·296	0·002	0·024	0·024	Nil
2	A	2·6	17·8	0·056	0·056	Nil	0·326	0·326	Nil	0·022	0·022	Nil
7	C	3·43	27·5	0·073	0·069	0·004	0·265	0·252	0·013	0·021	0·020	0·001
8	C	3·3	24·8	0·077	0·070	0·007	0·313	0·284	0·029	0·023	0·021	0·002
					Mean values		**0·301**	**0·290**	**0·011**			
9	Inanition	2·5	20·8	0·070	0·053	0·017	0·335	0·212	0·123	0·028	0·021	0·007
10	Inanition	2·5	23·8	0·066	0·054	0·012	0·275	0·226	0·049	0·026	0·022	0·004

The diet as in the case of rabbits appears to have no influence on the kidney cholesterol. The average values for normal animals are not very dissimilar from the mean of those given by Windaus [1910] for normal human kidneys, viz. 0·24 free and 0·02 ester per cent. In the case of the starved animals the ester cholesterol shows a marked increase, which Ellis and Gardner also showed to be the case in the rabbit. These high ester values recall the high ester value found by Windaus in human pathological kidneys.

Lung. The results are given in Table VIII.

TABLE VIII.

No. of cat	Diet	Wt. of organ taken for analysis, in g.	Percentages of cholesterol		
			Total	Free	Ester
1	A	17·1	0·416	0·295	0·121
2	A	15·6	0·361	0·296	0·065
7	C	17·5	0·435	0·247	0·188
8	C	11·4	0·453	0·270	0·183
9	Inanition	13·4	0·445	0·413	0·032
10	Inanition	16·5	0·376	0·376	Nil

The cholesterol content, as one would expect, appears to have nothing to do with diet. The values are of much the same order as those given for rabbits by Ellis and Gardner, viz. average of six animals, free 0·442 and ester 0·053. The ester cholesterol, as in the case of rabbits, is very variable.

Brain. The brains of cats 1 and 2 on diet A, 7 and 8 on diet C and 9 and 10 in a state of inanition, were examined with the following results:— 3·05, 2·65, 2·94, 2·91, 2·33 and 2·06 per cent. In no case was any ester found. The average is 2·66. For rabbits, Ellis and Gardner give as an average of eight experiments 2·32, with a variation of 2·02–2·88, and in no case was there any evidence of the presence of esters.

Bile. Attempts were made to estimate the cholesterol content of the bile of the cats under different diets and during starvation, but the contents of the gall-bladder were so small that it was difficult to estimate the cholesterol with any degree of accuracy. We give however the results for what they are worth in the following Table IX.

TABLE IX.

No. of cat	Diet	Wt. of bile in gall-bladder, in g.	Percentage of cholesterol		
			Total	Free	Ester
1	A	0·96	0·56	0·42	0·14
2	A	1·48	0·28	0·28	Nil
7	C	0·61	0·36	0·34	0·02
8	C	1·30	0·61	0·61	Nil
9	Inanition	2·53	0·25	0·03	0·22
10	Inanition	1·62	3·01	3·01	Nil

The results of the experiments on diets A and C do not indicate any connexion between either the actual amount of cholesterol in the bile or the percentage content and the diet. In the case of cat 10 there was a relatively high percentage of cholesterol in the bile, and this appeared to be all or mainly in the free state. This is in agreement with the observations of Ellis and Gardner on starving rabbits. On the other hand in the case of cat 9, also in a state of starvation, both the actual amount found and the percentage are of much the same order as in the fed animals, but it was nearly all found in the ester condition.

GENERAL CONCLUSIONS.

The results obtained in these experiments are strikingly similar to those obtained by Ellis and Gardner on the rabbit, and are in agreement with the hypothesis advanced at the beginning of this paper, viz. that cholesterol is a constituent constantly present in all cells, and that when these cells are broken down in the life process the cholesterol is not excreted as a waste product, but is utilised in the formation of new cells. A function of the liver is to break down dead cells, e.g. blood corpuscles, and eliminate their cholesterol in the bile. After the bile has been passed into the intestine in the process of digestion, the cholesterol is reabsorbed, possibly in the form of esters, along with the bile salts and is carried in the blood stream to the various centres and tissues for reincorporation into the constitution of new cells. Waste of cholesterol is made up from that taken in with the food.

REFERENCES.

Albrecht and Weltmann (1911), *Wiener med. Wochensch.* No. 14.
Bondzynski (1896), *Ber.* **29**, 276.
—— and v. Humnicki (1896), *Zeitsch. physiol. Chem.* **22**, 396.
Dorée (1909), *Biochem. J.* **4**, 72.
Dorée and Gardner (1908, 1), *Proc. Roy. Soc.* B, **80**, 212.
—— —— (1908, 2), *Proc. Roy. Soc.* B, **80**, 227.
—— —— (1909), *Proc. Roy. Soc.* B, **81**, 109.
Ellis and Gardner (1908), *J. Physiol.* **38**, *Proc.* i.
—— —— (1909, 1), *Proc. Roy. Soc.* B, **81**, 129.
—— —— (1909, 2), *Proc. Roy. Soc.* B, **81**, 505.
—— —— (1912, 1), *Proc. Roy. Soc.* B, **84**, 461.
—— —— (1912, 2), *Proc. Roy. Soc.* B, **85**, 385.
—— —— (1912, 3), *Proc. Roy. Soc.* B, **86**, 13.
Fraser and Gardner (1909), *Proc. Roy. Soc.* B, **81**, 230.
—— —— (1910), *Proc. Roy. Soc.* B, **82**, 559.
Goodman (1907), *Beiträge*, **9**, 91.
Grigaut (1913), *Le Cycle de la Cholestérinémie*.
Harley and Barratt (1903), *J. Physiol.* **29**, 341.
Hueck (1911), *Münchener med. Wochensch.* 2588.
Kosumoto (1908, 1), *Biochem. Zeitsch.* **13**, 354.
—— (1908, 2), *Biochem. Zeitsch.* **14**, 416.
Landau (1913), *Deut. med. Wochensch.* **12**, 1.
Müller (1900), *Zeitsch. physiol. Chem.* **29**, 129.
Pribram (1906), *Biochem. Zeitsch.* **1**, 414.
Wacker (1912), *Zeitsch. physiol. Chem.* **80**, 381.
Windaus (1909), *Ber.* **42**, 238.
—— (1910), *Zeitsch. physiol. Chem.* **65**, 110.

LV. ON THE OXIDATION OF COPROSTEROL AND COPROSTANONE.

PART I.

By JOHN ADDYMAN GARDNER and WILLIAM GODDEN.

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Dorée and Gardner [1908] showed that coprosterol, $C_{27}H_{48}O$, was readily oxidised on treatment with the theoretical quantity of chromic acid with the production of the corresponding ketone, coprostanone, $C_{27}H_{46}O$. This coprostanone crystallised in glistening leaves which, under the microscope, appeared to consist of thin plates, generally square, with one or all of the corners slightly truncated. It melted at 62–63° to a clear liquid. It yielded an amorphous semicarbazone, melting at 192°, and an amorphous oxime, melting at 71°. On treatment with phenylhydrazine it behaved in an abnormal manner and yielded a crystalline derivative, melting at 192°, which was subsequently shown by Dorée [1909] to be coprosteryl-carbazole, formed from the coprostanone-phenylhydrazone with elimination of ammonia. As the yield of pure coprostanone in the above preparation was only 60 %, it appeared desirable to submit the reaction to further investigation.

Oxidation of coprosterol by chromic acid. To make sure of oxidising the whole of the coprosterol, a considerable excess of chromic acid was used—three times the amount taken by Dorée and Gardner.

10 g. coprosterol dissolved in 130 cc. of glacial acetic acid, and heated to 70°, were treated in the course of 1 hour with a solution of 8·5 g. chromic anhydride in a little dilute acetic acid, the temperature being maintained by the heat of the reaction. After adding the chromic acid, the liquid was heated on the water bath for one hour, and then diluted with water. The bulk of the acetic acid was next neutralised with sodium carbonate, and the still strongly acid liquor was thoroughly extracted with ether. The ethereal solution was now thoroughly extracted with dilute caustic soda, and on evaporating the ether nearly pure coprostanone was obtained in a yield in all experiments of about 70 per cent.

The caustic soda extracts on acidification with hydrochloric acid gave a white crystalline precipitate of an acid mixed with an oily substance. The oil was got rid of by washing with petroleum ether, in which the crystals were insoluble. During the extraction of the ethereal solution of the oxidation product with caustic soda a small quantity of an insoluble basic chromium salt also separated, and this on treatment with hot hydrochloric acid yielded a further quantity of the crystalline acid. This acid is soluble in ether, but only difficultly soluble in benzene and in chloroform. It is sparingly soluble in hot acetone and somewhat more readily in boiling ethyl acetate or alcohol. It can be readily purified by recrystallisation from a mixture of ether and acetone or from ethyl acetate. It usually separates as a fine powder, and under the microscope appears in the form of well-defined square rods. It melts at 247° to a clear liquid, without any decomposition. For analysis the substance was dried in vacuo. It proved rather difficult to burn.

$$(1) \quad 0.2054 \text{ g.} \; ; \quad 0.5604 \; CO_2 \; ; \quad 0.1934 \; H_2O.$$
$$(2) \quad 0.2433 \text{ g.} \; ; \quad 0.6628 \; CO_2 \; ; \quad 0.2358 \; H_2O.$$

Found   (1) $C = 74.41$; (2) 74.30.   Calc. for $C_{27}H_{44}O_4$, $C = 74.94$; $C_{27}H_{46}O_4$, $C = 74.59$.
                $H = 10.46$; 10.76. $H = 10.26$; $H = 10.67$.

In other combustions in which the carbon came low, the hydrogen was 10.38, 10.23 and 10.63.

Sodium salt. On titration with caustic soda in alcoholic solution 0.6304 g. required 14.43 cc. $N/5$ NaOH. With another specimen 0.3134 g. required 7.24 cc. $N/5$ NaOH. Mol. wt. found (1) 436.8. (2) 432.8. Calc. for $C_{27}H_{46}O_4$, 434.

Ammonium salt. The acid dissolved in strong ammonia giving a soapy solution. The bulk of the free ammonia was boiled off, and on cooling the liquid set to a perfectly clear stiff jelly. The jelly dried up in vacuo, with gradual evolution of ammonia, to a white amorphous mass, which dissolved in hot water giving a slightly opalescent soapy solution. For analysis the substance, dried finally at 100°, was decomposed by excess of standard acid, filtered and the acid titrated back with soda. 0.3157 g. was decomposed with 25 cc. $N/5$ sulphuric acid and the excess of acid required 42.5 cc. soda. 25 cc. $N/5$ acid $= 49.2$ soda; percentage of $N = 3.19$. Calc. for $C_{27}H_{49}O_4N = 3.1$ %.

Silver salt. This was prepared by precipitating a neutral solution of the sodium salt with silver nitrate. The white precipitate was well washed with water, in which it was quite insoluble, and dried at 100°.

$$(1) \quad 0.298 \text{ gave on ignition } 0.0998 \text{ g. of silver.}$$
$$(2) \quad 0.1467 \quad ,, \qquad ,, \qquad 0.0489 \quad ,, \qquad ,,$$

Mol. wt. of acid found   (1) 430.6.   Calc. for $C_{27}H_{46}O_4$, 434.
                           (2) 433.8.

From the analysis of these salts and the combustions of the acid itself, the acid appears to be a dibasic acid of the formula $C_{27}H_{46}O_4$.

The oil formed along with this acid and dissolved away by petroleum ether as described above, would not crystallise on long standing. It was an acid and formed an insoluble barium salt on precipitating the solution in ammonia with barium chloride. This substance has not yet been further investigated.

Oxidation of coprostanone by chromic acid. In order to ascertain whether the acid $C_{27}H_{46}O_4$ was a bye-product in the preparation of coprostanone, or formed by the further oxidation of the coprostanone by the excess of chromic acid, one gram of coprostanone was dissolved in glacial acetic acid and heated to 70° with 0·35 gram of chromic anhydride dissolved in dilute acetic acid until all the red colour had gone. It was then heated to boiling, cooled and poured into water. The turbid liquid was extracted with ether, and on evaporating the ether a green oil containing chromium was obtained. This was freed from chromium by warming with hydrochloric acid on the water bath. The liquid was then extracted with ether, the ethereal solution evaporated and the brown oil left taken up with petroleum ether. The petroleum was allowed to evaporate spontaneously and left a brown oil, which on long standing in a desiccator deposited some crystalline matter. The oil was dissolved away by petroleum ether, and the crystalline matter recrystallised from hot ethyl acetate. It melted at 247° and was identical with the acid described above. The yield was however small. The oil dissolved by the petroleum was acid in character and formed an insoluble barium salt.

Oxidation of coprostanone by ammonium persulphate. As the yield of acid $C_{27}H_{46}O_4$ was small by both methods of preparation, we thought it desirable to try other oxidising agents and ultimately selected ammonium persulphate. This reagent is known to act on cyclic ketones [Baeyer and Villiger, 1899] breaking the ring and changing them to hydroxy-acids or their lactones; and we hoped in this way to break the ring of the coprostanone at the CO group and obtain a hydroxy-acid which might on further oxidation give the dibasic acid $C_{27}H_{46}O_4$. 10 g. coprostanone were dissolved in 375 cc. of glacial acetic acid, and to the solution were added 10 g. ammonium persulphate, dissolved in the least possible amount of water and diluted with an equal volume of glacial acetic acid. The mixture was then heated on a water bath and another 10 g. of powdered ammonium persulphate gradually added. The heating was continued for four hours, during which gas was slowly evolved. This gas was mainly oxygen from the persulphate, but contained some carbon dioxide. After standing several days the liquid was

diluted with an equal volume of water and thoroughly extracted with ether. The ethereal solution was repeatedly shaken with water to get rid of the bulk of the dissolved acetic acid, and then extracted with 10 % caustic soda solution several times, until a sample of the alkaline extract no longer gave a precipitate on acidification. The soda solution on shaking with the ethereal extract turned dark brownish red in colour. The various caustic soda extracts were mixed and on standing overnight bulky gelatinous clots of a colourless sodium salt separated out (A). These were somewhat difficult to filter, but it was found possible to syphon off the greater portion of the mother liquor. The mother liquors and filtrates, which were now pale yellow, on acidification with hydrochloric acid gave an amorphous precipitate of an acid substance (B). The ethereal solution, freed from acid matter, gave on evaporation a small quantity of a neutral substance (C).

Examination of (A). The clots were readily soluble in hot water giving a clear soapy solution, from which they separated on cooling, but more readily if the solution was made strongly alkaline. On acidifying the aqueous solution with hydrochloric acid a crystalline precipitate was thrown down which, in the preparation described, amounted to about 65 % of the weight of the coprostanone taken. In different preparations, however, the relative amounts of (A), (B) and (C) varied considerably with the conditions of reaction.

A small quantity of the clotted matter was filtered on the pump, washed with cold water to get rid of excess of alkali and further purified by means of alcohol. It was obtained as a white powder perfectly soluble in hot water. For analysis it was dissolved in water and precipitated by a measured quantity of standard acid. The precipitate was filtered, well washed and the filtrates titrated with alkali.

0·2488 g. was found to contain sodium equivalent to 6·1 cc. N/10 caustic soda; percentage sodium = 5·64. Calc. for $C_{27}H_{47}O_3Na = 5·21$.

The crystalline precipitate obtained on acidifying the aqueous solution of this sodium salt can be separated into two substances by fractional crystallisation from ethyl acetate or better from alcohol. The less soluble substance, present in small quantity, crystallised in thin plates and after several crystallisations melted at 183–184°. The more soluble substance, constituting the bulk of the material, crystallised in minute needles and melted at 157–158°.

Substance melting at 157–158°. This body was easily soluble in ethyl acetate, acetone and benzene, but somewhat less so in alcohol. It was sparingly soluble in petroleum ether, but more soluble in the fraction of

petroleum boiling at 156–195°. On combustion the following results were obtained :—

(1) 0·1549 ; 0·4568 CO_2 ; 0·1595 H_2O.
(2) 0·1571 ; 0·4625 ,, ; 0·1617 ,,
(3) 0·1640 ; 0·4834 ,, ; 0·1680 ,,
(4) 0·2557 ; 0·7503 ,, ; 0·2627 ,,

	I	II	III	IV	Calc. for $C_{27}H_{46}O_2$
C	80·42	80·29	80·40	80·02	C 80·52
H	11·44	11·43	11·38	11·41	H 11·50

Combustion does not however throw much light on the number of carbon atoms in the molecule, as decrease of the molecular weight by CH_2 makes very little difference in the percentage composition thus—

			C	H
$C_{27}H_{46}O_2$	...	...	80·52	11·50
$C_{26}H_{44}O_2$	...	...	80·34	11·42
$C_{25}H_{42}O_2$	...	...	80·14	11·31
$C_{24}H_{40}O_2$	...	...	79·93	11·19

The molecular weight was therefore determined by depression of the freezing point of benzene :—

(1) 0·2975 g. in 20 cc. benzene of sp. gr. 0·883, depression of freezing point 3·115° to 2·915°.
(2) 0·5993 g. in 15 cc. benzene of sp. gr. 0·883, depression of freezing point 3·115° to 2·565°.

Molecular weight found   I   412·7.   Calc. for $C_{27}H_{46}O_2$, 402·37.
II 403·1.

The substance did not go into solution on boiling for a short time with an aqueous solution of caustic soda, nor on shaking a solution in ether with either 10, 20 or 30 % caustic soda, though as originally obtained in the acetic acid solution it did so quite readily. It apparently contained no OH group, as it was quite unaffected by prolonged boiling with either acetic anhydride or acetyl chloride, and on pouring into water and extracting with ether it was recovered unchanged in both cases. It was probably a very stable lactone of a hydroxy-acid of the formula $C_{27}H_{48}O_3$.

Examination of (C). The neutral substance left in the ether after extraction with caustic soda, can be readily purified by recrystallisation from alcohol, or ethyl acetate, or petroleum ether. It melted at 183–184° and was identical with the substance of the same melting point obtained along with the lactone melting at 157–158°. This was proved by a mixed melting point. It crystallised in long thin plates, or glistening leaves, which under the microscope appeared to be rectangular plates, some square, some long. It proved difficult to burn and on combustion gave the following results :—

(1) 0·1197 ; 0·3514 CO$_2$; 0·1235 H$_2$O.
(2) 0·1720 ; 0·5052 „ ; 0·1672 „
(3) 0·1455 ; 0·4264 „ ; —
(4) 0·1580 ; 0·4615 „ ; 0·1594 „

	I	II	III	IV
C	80·06	80·11	79·92	79·66
H	11·46	10·80	—	11·21

0·8365 g. in 15 cc. benzene of sp. gr. 0·883, depression of freezing point 0·76°. Molecular weight found, 407 ; calc. for C$_{27}$H$_{46}$O$_2$, 402·37. The substance was not affected by shaking in ethereal solution with either 10, 20 or 30 % caustic soda solution.

The analyses threw no light on the relationship of the substance to the lactone melting at 157–158 . The molecular weight determination perhaps suggests that it is a stable lactone isomeric with the 157–158° body. It may however be a lactone of lower molecular weight produced by further oxidation, or possibly some derivative of a ketonic or diketonic nature. To test this 0·95 g. of the lactone (M.P. 156°) was dissolved in glacial acetic acid and 5·7 cc. of 10 % chromic acid solution added. The mixture was kept at 70° for half an hour, and then the temperature was raised to the boiling point for a few minutes. The liquid was then poured into cold water and the precipitated matter filtered off. The filtrate was extracted with ether and a further quantity of solid obtained, which was added to the precipitate. This precipitate was crystallised from alcohol from which it separated in needles and plates, melting at 169–171°. This weighed 0·38 g. or 40 % of the weight of the original substance. After several recrystallisations it melted at 183–184°. This was shown by a mixed melting point determination to be identical with the substance melting at 183–184° obtained from (C). The lactone (M.P. 157–158°) appears therefore to yield this neutral substance melting at 183–184° on oxidation. The latter body itself on similar treatment did not appear to be further oxidised, and was recovered unchanged. It did not appear to have ketonic properties for the following reasons : (1) sodium amalgam had no effect on the solution of the substance in moist ether in presence of sodium bicarbonate ; (2) in attempts to prepare a semicarbazone in the usual manner, the substance was recovered unchanged ; (3) on heating 1 mol. of the substance in alcoholic solution with 6 mols. of hydroxylamine hydrochloride and the equivalent amount of dry sodium acetate for 20 hours, the substance was recovered quantitatively and unchanged. On repeating experiment (3) however using caustic soda instead of sodium acetate, needle shaped crystals were obtained on evaporating the alcohol. These melted at 268–274°. This was not however an oxime as it contained only a trace of

nitrogen—under 1 %. The same or a very similar substance, melting at 264–286°, was also obtained on substituting phenylhydrazine hydrochloride for the hydroxylamine in the above experiment. This contained about 0·9 % of nitrogen. We had not sufficient material for the further investigation of these substances. It would seem probable however from these results that the substance of M.P. 183–184° is a stable lactone either isomeric with or containing less carbon than the lactone of M.P 157–158°.

Examination of substance (B). The acid precipitated on acidifying the alkaline solution was a light brown amorphous resin-like substance, readily soluble in alkalis giving a soapy solution. It was easily soluble in the common organic solvents, but would not crystallise. From petroleum ether it dried up to a brittle resin. It was dissolved in ammonia and reprecipitated by hydrochloric acid several times, and well washed. After drying it melted at 88° to a stiff paste and decomposed at 114°. The yield was only small.

Barium salts. The resinous acid was dissolved in ammonia, the excess of alkali exactly neutralised by hydrochloric acid and the solution precipitated with baryta water. The precipitate, after being well washed with water, dried to a light brown powder. This was readily soluble in chloroform, and when the solvent was allowed to evaporate spontaneously separated on the sides of the beaker in light brown brittle flakes. On analysis—

(1)   0·1367 ; 0·0327 BaSO$_4$.
(2)   0·2173 ; 0·0494   ,,
(3)   0·3541 ; 0·8468 CO$_2$; 0·3047 H$_2$O.

	Ba	C	H
I	14·11	—	—
II	13·50	—	—
III	—	65·22	9·56
Calc. for C$_{27}$H$_{45}$O$_4$Ba$_{1/2}$	13·68	64·53	9·03
Calc. for C$_{54}$H$_{93}$O$_7$Ba	13·83	65·25	9·64

The filtrate obtained after precipitating with baryta in the preparation of the above salt was acidified with hydrochloric acid and the small amount of amorphous resin precipitated was filtered and well washed. It was then dissolved in hot baryta water and the excess of baryta precipitated by carbon dioxide. The milky liquid was well boiled and the barium carbonate filtered off. The filtrate was evaporated to small bulk and left in a desiccator. Crystals gradually separated, which under the microscope appeared to be small plates. There was only sufficient for a barium estimation after drying at 100°.

0·1424 ; 0·0590 BaSO$_4$; Ba found = 24·39 ; Calc. for C$_{27}$H$_{44}$O$_4$Ba = 24·12 ; C$_{54}$H$_{93}$O$_7$Ba$_2$ = 24·35.

We hope shortly to be in a position to give a further account of the reactions of these various substances.

We take this opportunity of expressing our thanks to the Government Grant Committee of the Royal Society for assistance in carrying out this work.

REFERENCES.

Baeyer and Villiger (1899), *Ber.* **32**, 3625.
Dorée and Gardner (1908), *J. Chem. Soc.* **93**, 1629.
Dorée (1909), *J. Chem. Soc.* **95**, 653.

LVI. A NOTE ON A MODIFICATION OF TEICHMANN'S TEST FOR BLOOD.

By CLAUDE TREVINE SYMONS.

From the Government Analyst's Laboratory, Colombo, Ceylon.

For the identification of blood as such, several tests are in common use. But tropical conditions, such as are found in Ceylon, in many cases render some of these tests of little value. Hence I do not hesitate to put forward this modification of an old test, as I have found it to be very satisfactory.

The routine methods which may be adopted in cases of suspected blood stains are as follows :

I. *The benzidine test.* Benzidine is freshly dissolved in a small quantity of glacial acetic acid and hydrogen peroxide is added. This will give an *immediate* blue colour if it comes in contact with any blood. As, however, the immediate blue colour is produced by some other substances, the test merely serves to weed out a large number of stains which superficially resemble blood stains.

II. *The spectrum test.* In ordinary routine work it has been found that in suitable cases good results are obtained by soaking out the stain with ammonium hydrate and then adding ammonium sulphide to obtain the haemochromogen spectrum. But with very small stains there is often insufficient material to get the spectrum without special microscopical apparatus. In addition, when the stains have been long exposed to the tropical sun, they are apparently rendered so insoluble that the pigment is broken up before a solution can be obtained for the spectroscope. Further, in cases where the stain has been rusted on to iron and is not in large quantity, it has been found to be practically impossible to obtain a solution which will yield a spectrum.

III. *The corpuscle test.* This has been found to be of very doubtful value and very often impracticable in the case of old stains.

IV. *Teichmann's haemin crystal test.* This at first sight appears to provide a very convenient and reliable method of making a complete

identification of blood as such. In routine practice in the tropics, however, I understand that it has been largely discontinued, in consequence of the hitherto insuperable difficulties, which arise under certain conditions. As in the case of the spectrum test, old stains which have been exposed for a long time to air and sun in the tropics, and stains which are rusted on to iron, usually give negative results, even in cases where it is known that the stains were caused by blood.

Therefore I considered it of interest to examine the literature on the subject, and to evolve if possible some modifications which would give better results. The routine method in vogue had been to use glacial acetic acid with a trace of sodium chloride. Following the recommendations of various writers, I used, in place of sodium chloride, at first potassium iodide, and later sodium iodide with the glacial acetic acid. Good results were obtained with fairly fresh blood, but the results in the above mentioned obstinate cases were still unsatisfactory. So an attempt was made to substitute some other acid for the acetic acid.

Sulphuric, formic, valeric, butyric, and several other acids and combinations of acids and salts were tried without much success. Eventually strong lactic acid (sp. gr. 1·21 Kahlbaum) was tried with sodium iodide. This acid is mentioned in the literature as having been tried by Teichmann himself.

At first a very small fragment of the stain was placed in a drop of a solution of sodium iodide (approx. 5 %) in water, on a glass slide. This was carefully dried on an asbestos mat over a Bunsen flame. The cover glass was then placed over it and the lactic acid allowed to run under the cover glass, so as to cover the stain. The slide was then warmed on the asbestos mat and examined for crystals. Excellent results were obtained.

Later, solid sodium iodide, freshly prepared, was dissolved in the lactic acid to about 1 %. This solution becomes brown on keeping, but the change does not appear to impair its action, at any rate for some months. The fragment of stain is covered with the solution, and the cover glass is placed in position, and the whole slowly warmed as above to such a temperature that the solution is just about to boil under the cover glass. In most cases five minutes treatment is sufficient to produce crystals. If they have not then appeared, the heating must be continued; more solution must be allowed to run under the cover glass if necessary, but this is not often the case, as the lactic acid does not evaporate as rapidly as acetic acid. The resulting crystals differ very much in size in different cases, being usually rather small, but dark and perfectly characteristic.

By this method I have not failed to obtain crystals, in any case which

I have tried up to the present. Stains on filter paper which had been kept for three years exposed to the air, and were then exposed to direct sunlight for some hours, gave excellent results. An old stain, rusted on to a large iron knife, also gave perfectly clear results. These two failed to give any results with the other tests, except with benzidine.

One great advantage of the method is that only the very smallest fragment of stain is necessary. In fact it is essential for the production of good results that only a small amount of blood be taken. But it has also been found rather essential that the blood should not be very dilute. The smallest scraping from a solid article, or a shred of stained material about a millimetre long, is quite sufficient.

In using fresh blood it is advisable to dry the blood first.

Summary.

The benzidine test for blood stains is merely a preliminary test as it is not characteristic.

The spectrum and corpuscle tests fail in many cases, as does the ordinary method of using Teichmann's test. The use of a mixture of sodium iodide and lactic acid is recommended for the latter, in place of sodium chloride and acetic acid.

LVII. ON THE BEHAVIOUR OF AMYLASE IN THE PRESENCE OF A SPECIFIC PRECIPITATE.

By AGNES ELLEN PORTER,

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The complement-binding phenomenon which takes place in the presence of antigen and antibody has become of great practical importance in the recognition of disease. The difficulties of the test are great, owing to the fact that five different, chemically impure, and little known substances must be employed: antigen, antiserum, complement, amboceptor, and red corpuscles. The last three components are very troublesome to obtain, and it is not surprising that many efforts should be made to minimise this trouble, and to lessen the difficulties which are necessarily dependent on such a complicated process. The most attractive simplification would be one in which all these three components of the haemolytic system, viz. complement, amboceptor, and red corpuscles, are entirely done away with. This would be possible if some substance could be found which would become fixed, like complement, by the antigen-antibody mixture, but whose absence could be tested for more easily.

Like complement, animal ferments seem to be very rapidly absorbed by coagulated colloids and even by organic precipitates of different kinds. The ferment shows some selective action in this tendency. Pepsin absorbed into egg-white [Dauwe, 1905, p. 426] or pepsin, ptyalin, etc. into collodion [Porter, 1910, p. 382] can be recovered in some degree by a solution of the particular substance which they digest. As ferments can only be recognised through their effects, this possibility of partial recovery presents the chief objection to the use of ferments as substitutes for complement.

Most ferments are powerfully influenced by the presence of serum. Proteolytic ferments are inhibited by serum, probably because their attraction to the serum proteins, even in a dissolved condition, is already so great. On the other hand other ferments, such as starch-splitting ferments and lipases, are markedly accelerated by the presence of serum.

Hailer [1908, p. 280] first made the attempt of absorbing a ferment by means of a specific precipitate. He chose rennet, with sheep's serum as antigen, and the serum of a rabbit immunised against sheep's protein as antibody. This choice was perhaps unfortunate, as not only rennet but also antirennet have been described in serum [Fuld and Spiro, 1900, p. 141]. Also, as I have pointed out, rennet suffers very little from dilution, when the dilution has been freshly made. Rennet has indeed acted perfectly in my hands [1911, p. 394] at a dilution of 1/340,000. It can therefore be almost entirely absorbed without any evidence of the fact. On this account I thought it advisable to attempt the absorption with another ferment. Pepsin and trypsin are inactivated by serum; I therefore chose amylase. Szumowski [1898, p. 162] has proved that amylase is absorbed by fibrin. It is much less easily absorbed by serum proteins in their natural state, as serum is itself amylolytic. The advantages of the ferment were therefore as follows:

(1) It is present in serum, so that, were the absorption successful, no further addition of ferment to the antiserum would be required.

(2) It is apparently not absorbed by serum proteins in their normal state, i.e. before the union of antigen and antibody.

(3) It loses rapidly on dilution, so that even a small loss could be measured.

METHOD.

Ferment, guinea-pig-serum, saliva, and taka-diastase.

Antibody, serum of rabbits immunised against egg-white or against horse-serum.

Antigen, egg-white or horse-serum.

The ferment, in as small bulk as possible, i.e. 0·05 cc. saliva, or 0·15 cc. diluted taka-diastase, was added to 0·15 cc. immune or normal serum, and 0·15 cc. of the various dilutions of egg-white or horse-serum, the mixture being left at room temperature overnight. Next morning 3 cc. of a 1 % soluble starch solution were added, and the tubes were placed at 37° for 5 to 20 minutes, or in the case of guinea-pig-serum for a half to one and a half hours. The mixtures were then tested for sugar by means of Fehling's solution, and for dextrins by means of iodine. On account of the protein present, use was made of the dialysed iron method, which had the double advantage of removing both protein and ferment. When the mixtures were taken from the incubator, they were made up to a bulk of 5 or 6 cc. with physiological saline solution, and 0·5 cc. saturated salt solution, and 0·5 cc.

B.P. Liquor Ferri Dialysatus were added to each and the resulting precipitate immediately filtered off. A measured amount of the perfectly clear and inactive filtrate was then tested quantitatively for sugar. The colour given by the iodine test in the filtrate was clear and permanent.

The difference between the effect of immune and normal serum on amylase in the presence of antigen was very slight, but on account of the accuracy of the method and the number of times the experiment has been repeated, it may be taken as the expression of a genuine though very partial absorption.

Absorption experiment.

A. *Immune serum.* Rabbit immunised against egg-white.
(To each tube were added 0·05 cc. saliva, 0·15 cc. anti-egg-serum, 0·15 cc. diluted egg-white. method as above.)

Dilution of egg-white	Time at 37° with starch	Cc. of Fehling sol.	Iodine solution 0·5 cc.
1/10	10 minutes	0·2	Dark mauve.
1/50	,,	0·225	..
1/100	,,	0·225	,,
1/250	,,	0·25	,,
1/500	,,	0·25	Light mauve.
NaCl	,,	0·3	Pale pink.
1/10	20 minutes	0·25	Dark mauve.
1/50	,,	0·25	Medium mauve.
1/100	,,	0·25	,, ,,
1/250	,,	0·275	Medium light mauve.
1/500	,,	0·3	Light mauve.
NaCl	,,	0·35	Very pale.

B. *Normal rabbit-serum.*
(To each tube were added 0·05 cc. saliva, 0·15 cc. normal serum, 0·15 cc. diluted egg-white. method as above.)

1/10 to 1/500	10 minutes	0·3	All pale.
NaCl	,,	0·3	Pale.
1/10 to 1/500	20 minutes	0·375	..
NaCl	,,	0·375	..

When serum is used as antigen a curious phenomenon may be noticed. In this case all three ingredients, antigen, antibody, and saliva, contain amylase from three separate species, and this gives rise to an acceleration which is decidedly beyond the sum of the three separate activities. Although the antiserum was heated at 56° to reduce its amylolytic power, it was still able to exert an accelerating influence. In the following experiment, where the antigen is horse-serum, acceleration is to be seen and must be allowed for. In spite of it, absorption can be observed if the "immune" and "normal" columns are compared.

A. *Rabbit-serum*, immune to horse-serum.

(To each tube were added 0·05 cc. saliva, 0·15 cc. anti-horse-serum, 0·15 cc. diluted horse-serum, method as above.)

Dilution of horse-serum	Time at 37° with starch	Ccs of Fehling sol.	Colouration with iodine solution
1/10	10 minutes	0·5	Red mauve.
1/50	,,	0·45	,,
1/100	,,	0·4	,,
1/250	,,	0·4	Same, but deeper.
1/500	,,	0·35	Mauve.
NaCl	,,	0·35	,,
1/10	20 minutes	0·5	Pale pink.
1/50	,,	0·5	,,
1/100	,,	0·45	,,
1/250	,,	0·4	Mauve.
1/500	,,	0·35	,,
NaCl	,,	0·35	..

B. *Normal rabbit-serum.*

(To each tube were added 0·05 cc. saliva, 0·15 cc. normal rabbit-serum, 0·15 cc. diluted horse-serum, method as above.)

1/10	10 minutes	0·55	Colourless.
1/50	,,	0·55	,,
1/100	,,	0·5	,,
1/250	,,	0·4	Pale.
1/500	,,	0·35	Mauve.
NaCl	,,	0·35	,,
1/10	20 minutes	0·6	Colourless.
1/50	,,	0·6	,,
1/100	,,	0·55	,,
1/250	,,	0·45	,,
1/500	,,	0·4	Pale.
NaCl	,,	0·35	Mauve.

Taka-diastase displayed no tendency whatsoever to become absorbed by a specific precipitate. This is interesting, as I have noticed before [1910, p. 386] that while ptyalin was inactivated by collodion membranes in a day, taka-diastase was hardly affected in a month's time.

BEHAVIOUR OF SERUM AMYLASE ON PRECIPITATION WITH CARBON DIOXIDE.

Fresh guinea-pig-serum, diluted to 1/10 in water, was saturated with carbon dioxide, and the globulins separated and redissolved in saline solution, after centrifuging. The upper fluid taken from the centrifuge had its isotonicity restored by sodium chloride, and both portions were tested for amylase. The ferment was found to be almost unaffected by the precipitation, practically the whole ferment remaining free in the upper fluid.

Time 1½ hours	Cc. of Fehling sol.	Colouration with iodine
1 cc. serum 1/10 + 1 cc. NaCl 0·85 %	0·5	Brown.
1 cc. precipitate (1/10) + 1 cc. NaCl 0·85 %	0·05	Dull, dark.
1 cc. upper fluid + 1 cc. NaCl 0·85 %	0·4	Brown.
1 cc. serum 1/10 + 1 cc. precipitate	0·475	,,
1 cc. serum 1/10 + 1 cc. upper fluid	0·65	Colourless.
1 cc. precipitate + 1 cc. upper fluid	0·425	Brown.

Although these experiments have been attended with little success, I am venturing to record them as of some small theoretic interest, and partly to recommend a good method for testing amylolytic action in the presence of serum.

REFERENCES.

Dauwe (1905), *Beiträge*, **6**, 426.
Fuld and Spiro (1900), *Zeitsch. Physiol*. **31**, 132.
Hailer (1908), *Arbeit. kaiserl. Gesund.-Amt*. **29**, 277.
Porter (1910), *Quart. J. exp. Physiol*. **3**, 375.
—— (1911), *J. Physiol*. **42**, 389.
Szumowski (1898), *Arch. Physiol*. 5ᵉ série, 160.

LVIII. THE GALACTOSIDES OF THE BRAIN. I.

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Nearly forty years ago Thudichum [1874] recognised the existence in the brain of substances analogous in their constitution to the vegetable glucosides. He distinguished two principal representatives of this class which he called phrenosin and kerasin. Phrenosin being relatively easy to prepare in sufficient amount for further study, Thudichum succeeded in elucidating its constitution by an investigation of its hydrolytic cleavage products. The carbohydrate obtained on hydrolysis of phrenosin was originally called "cerebrose" by its discoverer, but was identified later with galactose [Thierfelder, 1890; Brown and Morris, 1890]. The group name "cerebroside," in analogy to "glucoside," was introduced by Thudichum on the assumption that cerebrose was the typical carbohydrate of the brain, but later on he himself adopted the name cerebro-galactoside. In view of the fact that the occurrence of these substances is not limited to the brain[1], the use of the general name "galactoside" seems to be preferable to the special term "cerebroside."

The work of subsequent investigators was limited, until quite recently, to the preparation by modified methods of the substances first obtained by Thudichum, without contributing to the knowledge of their cleavage products. Unfortunately a great confusion in the nomenclature of these substances has resulted from the failure of earlier workers to co-ordinate their results with each other and with those of Thudichum. It seems that too much importance was attached to minor differences in the figures of elementary analysis and to variations in the melting points of these substances of high molecular weight; it was mainly on this basis that new names like pseudo-cerebrin, cerebrin, homocerebrin, cerebron were introduced for substances, which from the method of their preparation were nothing else than Thudichum's original phrenosin and kerasin, or mixtures of the two.

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[1] Similar substances have since been obtained from pus (Kossel and Freitag), from blood (Bang and Forssmann), from adrenals (Rosenheim and Tebb), and also from certain mushrooms (Zellner), etc.

In the course of a prolonged investigation of these substances, carried out during the last six years, the author has been able to confirm Thudichum's main observations and has worked out a new method for the preparation of the galactosides from brain. The complete separation of the galactoside mixture into its two principal constituents, phrenosin and kerasin, was made possible for the first time by the help of a new physical test, depending on the behaviour of these substances under the polarising microscope, which will be described later.

The investigation of the optical activity of the two substances brought out an interesting difference between them. Whilst phrenosin, in confirmation of the previous observations of Thierfelder and Kitagawa [1906], proved to be dextro-rotatory, it was found that kerasin, when completely separated from all dextro-rotatory phrenosin, possessed a laevo-rotation. Levene and Jacobs [1912, 2] and subsequently Thierfelder [1913] have since described as inactive a "kerasin fraction" prepared by different methods and evidently not completely freed from phrenosin.

The two products were further subjected to hydrolysis and all the cleavage products were identified. As far as phrenosin is concerned, the results agreed with Thudichum's observations which had already been confirmed in the main outlines by Thierfelder's work [1904, 1905].

The complete hydrolysis of kerasin had hitherto not been carried out and its relationship to phrenosin had remained unexplained. In its elementary composition it agrees closely with phrenosin and yet it differs widely from it in appearance, solubility and other physical constants. Thudichum suggested that the essential difference between it and phrenosin might be due to a difference in the fatty acid radicle. Neither the fatty acid nor the carbohydrate nor the base had been isolated or identified by Thudichum. Levene and Jacobs [1912, 2] recently advanced the hypothesis that the two substances represented optical isomers.

My own results show that Thudichum's suggestion was correct and that kerasin contains a different fatty acid from phrenosin. On hydrolysis of kerasin, purified as far as possible, I obtained as the only fatty acid one of the composition $C_{24}H_{48}O_2$, which I was able to identify with lignoceric acid [1913]. The carbohydrate was found identical with d-galactose and the base with sphingosine, as was to be expected from Thudichum's preliminary work.

Phrenosin, on the other hand, gives rise to the optically active hydroxy-acid $C_{25}H_{50}O_3$, as well as to d-galactose and sphingosine[1].

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[1] Thierfelder [1904] was the first to show that the fatty acid obtained by Thudichum from phrenosin represented a hydroxy-acid. Levene and Jacobs [1912, 1] satisfactorily explained the

In connection with this work the question was investigated in which form the galactosides occur in the brain, and it is proposed to deal with this fundamental question first, leaving the description of the methods used for the separation of phrenosin and kerasin, as well as for their purification and hydrolysis, for a subsequent communication.

I. The galactosides exist in the brain in the free state.

Previous investigations leave the question undecided whether the galactosides occur preformed in the brain or whether they are split off from more complex substances during the process of preparation. Whilst some recent authors incline to the view that part at least exist preformed, others are of the opinion that, as only small quantities are obtainable directly from the brain without drastic methods, they owe their origin to the partial decomposition of a preformed complex substance. No experimental proof for either view has been brought forward.

It is evident that this question cannot be decided by the use of those methods hitherto employed for the preparation of the galactosides, in which either baryta is used for the removal of the phosphatides, or in which solvents like alcohol are used at their boiling temperature. In both cases a decomposition of a preformed combination is possible. It seemed feasible, however, to obtain conclusive evidence on this point by the use of an inert solvent which dissolves galactosides in the cold, and in which phosphatides and sulphatides are insoluble. Such a solvent was found by Rosenheim and Tebb [1910] in pyridine.

In applying the pyridine method directly to brain, it is preferable previously to remove water and cholesterol by means of cold acetone [Rosenheim, 1906], and the unsaturated phosphatides, lecithin and kephalin, by means of ether or petroleum ether. As the same method was used subsequently for the isolation of the galactosides, the procedure adopted may be described here in detail.

differences in melting point of Thudichum's and Thierfelder's acid by showing that the acid existed in two isomeric modifications, of which the optically inactive one melts at 82–85° (Thudichum's "neurostearic" acid), whilst the dextro-rotatory form melts at 106–108° (Thierfelder's "cerebronic" acid). From a "kerasin fraction" Thierfelder [1913] has recently obtained an acid of the composition $C_{24}H_{48}O_2$; Levene [1913] has since identified a similar acid as lignoceric acid, which he isolated on hydrolysis of a "cerebrin fraction." Both Thierfelder's and Levene's fractions were optically inactive and contained therefore probably dextro-rotatory phrenosin admixed with the laevo-rotatory kerasin. Thierfelder, indeed, obtained 6 % of cerebronic acid, the typical acid of phrenosin, on hydrolysis of his "kerasin fraction." The carbohydrate had not been identified by either of these workers.

10 kg. of finely minced ox brain were suspended in 10 litres of acetone and allowed to stand with frequent stirring for 24 hours at room temperature. The watery acetone extract was decanted and the brain pulp strained through several layers of fine muslin. At least six subsequent extractions with sufficient acetone to cover the tissue were made, until the last extract on evaporation yielded only an inappreciable amount of cholesterol. The total quantity of crude cholesterol obtained from these extracts varies between 240–260 g., i.e. about 2·5 % of the fresh brain.

The tissue was next spread in a thin layer on large glass plates, gently warmed from below, and freed from acetone by means of an air current (electric fan).

The dry, somewhat waxy powder was now subjected to extraction with cold petroleum ether. Five to six extractions were usually found sufficient to remove the unsaturated phosphatides, which may be obtained from the extracts by the usual methods. After the removal of the petroleum ether by an air current in the way described above, the tissue was passed through an Excelsior mill and was thus obtained as a fine cream-coloured powder, ready for the pyridine treatment. 10 kg. of fresh brain yield on the average 1300 g. (from 1200–1400 g.) of this powder, which seems to keep indefinitely in this condition.

For the preparation of the galactosides it was found convenient to work up 500 g. of this powder at a time. This quantity was covered with 1500 cc. of pyridine (B.P. 115°) and after having been warmed to 45° by being kept for about twenty minutes in a water-bath at 50°, it was rapidly cooled to room temperature. Although the galactosides are readily soluble in cold pyridine, the initial warming is necessary in order to allow the solvent to penetrate the tissue. Filtration proceeds easily by means of a large Buchner funnel. From their pyridine solution the galactosides were obtained by pouring it into 3–4 volumes of acetone[1]. A bulky white precipitate is formed, from which the supernatant fluid can be easily decanted off after standing some time. The mother liquor deposits still further on cooling on ice. In the earlier experiments this deposit was filtered off separately, but as the amount was found to be very small, the pyridine-acetone mixture was cooled down directly to 0° in the later experiments.

The filtration and washing of the mixed galactosides under pressure proceeds very slowly and it is therefore advisable to filter through a plain filter. After thorough washing with acetone, the precipitate is suspended in acetone and only then filtered under pressure. After being dried in vacuo,

[1] The pyridine solution may, of course, be previously concentrated by distillation in vacuo.

the powder is extracted with ether in a Soxhlet, in order to remove the last traces of ether-soluble phosphatides.

The crude galactosides are thus obtained as a slightly yellowish powder, the average yield amounting after two extractions to 205 g. from 10 kg. of moist brain, i.e. 2 per cent.

It is of interest to compare this experimental yield with the theoretical. Unfortunately there is at present a lack of a reliable quantitative method for the estimation of galactosides and the data available in the literature as to the percentage of galactosides in normal brain are correspondingly scanty. Probably the method worked out recently by Lorrain Smith and Mair [1913], in which the galactosides are weighed as such, gives truer results than the older methods in which the reducing power of hydrolysed brain extracts served as a basis of calculation. Lorrain Smith and Mair state their results in percentages of the chloroform extract of dried brain. Taking the mean water content of brain as 78 %, I have calculated from their results the percentages of galactosides as 7·3 % in dry and 1·6 % in fresh normal human brain. This result agrees well with the experimental yield of 9·8 % in dry and 2 % in fresh brain, as stated above, if we consider that these figure refer to the crude product.

After having been recrystallised twice from 15 volumes of an alcohol-chloroform mixture (1:2) the substance was obtained as a white powder, which contained 1·68 % of nitrogen and only a very small percentage of phosphorus (0·08 %).

> 0·3000 g.; 2·36 cc. N/10 KOH by Neumann's method.
> 0·4893 g.; 5·86 cc. N/10 KOH by Kjeldahl's method.

The product represents, as will be shown in a subsequent communication, a mixture of at least two substances. For the purpose of the present investigation it was considered sufficient to show that it consists essentially of galactosides, and the substance was therefore subjected to hydrolysis without any further purification.

Method of estimation of the products of hydrolysis.

Hydrolysis was carried out in methyl alcohol solution with sulphuric acid, and the cleavage products estimated in a way similar to that described by Thierfelder [1905] for "cerebron." Galactose was estimated polarimetrically and the fatty acids (as esters) and bases (as sulphates) were collected as carefully as possible and weighed.

1 g. of the substance was dissolved in 50 cc. methyl alcohol containing

5 cc. concentrated sulphuric acid and boiled under a reflux condenser on a water bath for six hours. After standing overnight, white glittering scales of the esters (and free fatty acids) had crystallised out. The flask was kept in the ice-chest for some hours and the crystals were filtered off, washed with cold methyl alcohol, dried in vacuo and weighed. To the filtrate water was added, and the clear solution boiled for some time in order to hydrolyse the methyl galactoside formed during hydrolysis. The alcohol was evaporated on the water bath. During the concentration of the watery solution, oily droplets of the sulphates of the bases appeared, which solidified on cooling. The deposit was filtered, taken up in boiling alcohol and the solution evaporated in vacuo. The residue consisting of the sulphates of sphingosine (and dimethylsphingosine) was weighed after having been dried to constant weight in vacuo. The final filtrate was made up to 100 cc. and examined polarimetrically.

Previous experiments had shown us that the results of galactose estimations by the polarimeter agreed with those made by Kjeldahl's gravimetric method.

Experiment I. 1 g. substance gave 0·303 g. esters and 0·620 g. sulphates of bases. Galactose solution: actual rotation measured = + 0·33° (mean of six readings) in 2 dm. tube. Whence galactose = 20·39 °/₀ [see Landolt, 1892, p. 452].

Experiment II. 1 g. substance of a different preparation gave 0·280 g. esters. Galactose solution: actual rotation measured = + 0·34° in 2 dm. tube. Whence galactose = 21·01 °/₀.

d-Galactose was identified in the final solution by means of methylphenylhydrazine. The free mineral acid was neutralised with solid sodium acetate and methylphenylhydrazine was added. After standing at room temperature for some hours, crystals of the hydrazone settled down. They were filtered off and recrystallised from alcohol. The white crystals melted sharply at 191°. The melting point of galactose methylphenylhydrazone is given by Neuberg [1907] as 191° [see also Fränkel, 1910]. From another portion of the hydrolysate, galactose was prepared as such by the usual methods and identified as mucic acid by oxidation with nitric acid. The dry ammonium salt of the mucic acid thus prepared gave on heating a strong pyrrole reaction.

The results are given in the following table (page 610), which shows also for comparison the figures obtained by a similar method by Thierfelder from the hydrolysis of more or less purified galactosides.

It will be seen from these results that the cleavage products obtained on hydrolysis of the galactoside mixture show a close resemblance in their nature, as well as in their quantitative distribution, with those obtainable from the

	Galactoside mixture prepared by the pyridine method		Phrenosin[1] (cerebron)	Kerasin fraction
	I	II		
Nitrogen %	1·68	—	1·76	1·61
Galactose %	20·39	21·01	19·88	19·35
Esters + fatty acid %	30·3	28·0	38·7	29·6
Bases (as sulphates)	62·0	—	50·3	57·2

[1] The figures given represent the mean values calculated by me from Thierfelder's figures.

galactosides isolated by a different method. This result therefore seems to justify the conclusion that the product obtainable from brain by extraction with cold pyridine, consists practically entirely of galactosides. In their preparation any possible decomposition of a preformed complex substance has been carefully avoided by limiting the time of extraction to a minimum and by employing an inert solvent at a low temperature. As, further, the amount obtainable experimentally agrees very well with the theoretical yield, we must assume that the whole of the galactosides exist in the brain in the preformed state.

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SUMMARY.

(1) A new method for the preparation of galactosides from brain by means of pyridine is described.

(2) Evidence is brought forward to show that the galactosides exist in the brain entirely in the preformed condition.

REFERENCES.

Brown, Horace T. and Morris, G. H. (1890), J. Chem. Soc. 57, 57.
Fränkel, S. (1910), Biochem. Zeitsch. 26, 41.
Landolt, H. (1892), Das Optische Drehungsvermögen, Braunschweig.
Levene, P. A. and Jacobs, W. A. (1912, 1), J. Biol. Chem. 12, 381.
—— —— (1912, 2), J. Biol. Chem. 12, 389.
—— (1913), J. Biol. Chem. 15, 359.
Neuberg, C. (1907), Biochem. Zeitsch. 3, 531.
Rosenheim, O. and Tebb, M. C. (1910), J. Physiol. 40, Proceedings, i.
—— (1906), J. Physiol. 34, 104.
—— (1913), Trans. Internat. Congress of Med. Sect. II. 626.
Smith, J. Lorrain and Mair, W. (1913), J. Path. Bact. 17, 609.
Thierfelder, H. (1890), Zeitsch. physiol. Chem. 14, 209.
—— (1904), Zeitsch. physiol. Chem. 43, 21.
—— (1905), Zeitsch. physiol. Chem. 44, 366.
—— and Kitagawa, F. (1906), Zeitsch. physiol. Chem. 49, 288.
—— (1913), Zeitsch. physiol. Chem. 85, 35.
Thudichum, L. J. W. (1874), Reports of the Med. Off. of the Privy Council and Local Gvt. Board, New Series, No. III.
—— (1882), J. pr. Chem. 25, 19.
—— (1901), Chem. Konstitution d. Gehirns d. Mensch. u. d. Tiere, Tübingen.

LIX. THE ESTIMATION OF PYRUVIC ACID.

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(Received Nov. 11th, 1913.)

Pyruvic acid forms with phenylhydrazine a hydrazone by means of which, as Emil Fischer pointed out, one part of this acid in one thousand parts of water may be detected. The yield of hydrazone however is not quantitative and the attempts made by some authors to estimate pyruvic acid by weighing the phenylhydrazone precipitated have not proved satisfactory. Subsequently nitrophenylhydrazine was used as the precipitating reagent and by this means Neuberg and Karczag [1911] recovered 92 % of pyruvic acid from a 1 % solution, as the nitrophenylhydrazone. In estimating the amount of pyruvic acid in solutions containing 0·1 % and of still lower concentrations this method is however of very little value, since the error introduced by the appreciable solubility of the hydrazone becomes of increasing importance with increasing dilution. An investigation into the action of the tissues on dilute solutions of pyruvic acid which I had undertaken had temporarily to be abandoned since the apparent removal of the acid observed might have been explained by an increase in the amount of hydrazone held in solution, and even when controls of corresponding dilution were used the results were unsatisfactory.

The action of asymmetrical diphenylhydrazine was investigated and was found to present similar difficulties.

If the experimental errors found above were due to the solubility of the hydrazone, the determination of the amount of phenylhydrazine removed from the solution in combination as hydrazone should give satisfactory results.

Estimation of Phenylhydrazine.

Fischer [1878] showed that phenylhydrazine was oxidised by cold dilute Fehling's solution with evolution of nitrogen; benzene and aniline were

formed and cuprous oxide precipitated. Strache and Kitt [1892] estimated the volume of nitrogen liberated and showed that if boiling solutions were used, no aniline was formed and the whole of the nitrogen was liberated in the free state; under these conditions six molecules of cupric oxide were necessary to oxidise two molecules of phenylhydrazine, a mixture of benzene and phenol being obtained. The reaction may be represented as follows:

$$2C_6H_5 . HN . NH_2 + 3O = C_6H_6 + C_6H_5OH + 2N_2 + 2H_2O.$$

The benzene formed during the reaction exerts an appreciable influence on the vapour tension, a difficulty which Strache overcame by saturating the gas both with benzene and with water vapour and introducing the necessary corrections. Strache [1891, 1892] estimated ketones and aldehydes by allowing warm solutions of the carbonyl compound and phenylhydrazine to react and then measuring the excess of phenylhydrazine in the solution by determining the volume of nitrogen evolved when oxidised by boiling Fehling's solution.

The method is not very convenient and it would be preferable to estimate the cuprous oxide formed. By the above method, however, in working with tissue-extracts containing pyruvic acid, any sugar present would react with the boiling Fehling's solution. If, however, the cuprous oxide formed when the excess of phenylhydrazine reacts with Fehling's solution at air temperature be estimated, this difficulty can be obviated.

Experiments were therefore made in order to determine whether the amount of cuprous oxide precipitated by a certain weight of phenylhydrazine was constant.

The Fehling's solution was made up as in Bertrand's method for estimating glucose.

Solution I. Copper Sulphate crystals 40 grams per litre.
Solution II. Rochelle Salt 200 grams } per litre.
             Caustic Soda 150   „   }

5 cc. of a solution of phenylhydrazine containing 3·5236 g. in 100 cc. of 50 % acetic acid were diluted to 100 cc. with water, and allowed to stand at the ordinary temperature for 30 minutes: in nine experiments, 20 cc. of each of the Fehling's solutions, I and II made up as above, were added to 10 cc. of the diluted phenylhydrazine solution and the mixture allowed to stand at the ordinary temperature for times varying from half an hour to four hours and a half. The cuprous oxide formed was then filtered through a Gooch crucible, dissolved in ferric sulphate solution as in Bertrand's method for the estimation of glucose and the ferrous sulphate produced titrated with deci-normal permanganate solution.

Results.

Time	Cc. N/10 KMnO$_4$ equivalent to Cu$_2$O formed
30 minutes	6·0
60 ,,	6·05
90 ,,	6·0
150 ,,	6·05
150 ,,	6·0
180 ,,	6·0
210 ,,	6·0
240 ,,	5·95
270 ,,	5·95

The reaction between phenylhydrazine and Fehling's solution appears therefore to reach a definite stage of equilibrium within half an hour at the ordinary temperature, after which no further oxidation proceeds.

In three experiments, the following values were obtained:

1 cc. of N/10 KMnO$_4$ was equivalent to (1) 0·002962 gr. phenylhydrazine,

$$\qquad\qquad\qquad\qquad (2)\ 0·002962 \qquad\qquad ,,$$

$$\qquad\qquad\qquad\qquad (3)\ 0·002935 \qquad\qquad ,,$$

As the mean of these experiments therefore

1 cc. N/10 KMnO$_4$ is equivalent to 0·00295 g. C$_6$H$_5$NH.|NH$_2$.

It may be mentioned that in filtering the cuprous oxide from the cold Fehling's solution, it is advisable to filter only under a slight difference of pressure, as there is a tendency for the cuprous oxide to pass through the asbestos. If this happens the filtrate should be filtered through a clean Gooch crucible and the two results added together.

Estimation of Pyruvic Acid.

A specimen of Kahlbaum's pyruvic acid was distilled under diminished pressure and the fraction boiling at 77°–78° under a pressure of 15–20 mm. used for the estimation. The method adopted was as follows:

A solution of pyruvic acid was made up containing 1·5228 g. per 100 cc. Quantities of from 2 to 10 cc. of this solution were diluted to about 80 cc., 5 cc. of a solution of phenylhydrazine, approximately 4 %, added, and the mixture made up to 100 cc. and allowed to stand half an hour at the ordinary temperature; 5 cc. of the hydrazine solution were diluted to 100 cc. and allowed to stand for the same time. After half an hour the pyruvic hydrazone which had separated was filtered off and 10 cc. of each filtrate

added to 40 cc. of Fehling's solution. Ten cc. of the control phenyl-
hydrazine solution were similarly treated. The cuprous oxide was estimated
as above described.

Thus:

10 cc. phenylhydrazine solution require	6·00 N/10 KMnO₄

10 cc. phenylhydrazine solution require $\quad$ 6·00 N/10 $KMnO_4$
$\qquad\qquad\qquad\qquad\qquad\qquad\qquad$ 6·00 ,, ,,

10 cc. hydrazone filtrate require $\quad$ ... $\quad$ 2·75 ,, ,,
$\qquad\qquad\qquad\qquad\qquad\qquad\qquad$ 2·75 ,, ,,

3·25 cc. N/10 KMnO₄ are equivalent to $3·25 \times 0·00295$ g. phenylhydrazine.

$$C_6H_5HN . NH_2 + CH_3 . CO . COOH \rightarrow CH_3 . C (N_2HC_6H_5) . COOH.$$
$$108 \qquad\qquad 88$$
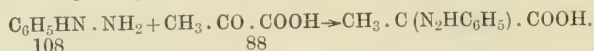
3·25 cc. N/10 KMnO₄ are equivalent to $\dfrac{88 \times 0·00295 \times 3·25}{108}$ g. pyruvic acid, and 10 cc. of the
diluted pyruvic solution contained 0·00780 g. pyruvic acid, and the original solution contained
1·556 %.

10 cc. of the diluted solution contained by weight 0·007614 g. and the original solution
1·5228 %.

The following table shows some of the results obtained:

Ten cc. of a solution of pyruvic acid contained:

(a) By weight	(b) By above method of estimation	Error
2·68 milligrams	2·57 milligrams	− 0·11 mgr.
3·19	2·15	− 1·04
3·57	2·51	− 1·06
5·36	5·14	− 0·22
6·28	6·57	+ 0·29
7·14	7·46	+ 0·32
7·61	7·76	+ 0·15
8·04	7·71	− 0·33
9·42	9·80	+ 0·38
9·58	9·20	+ 0·38
10·71	11·22	+ 0·51
10·71	10·34	− 0·37
11·97	12·31	+ 0·34
12·77	12·67	− 0·10
13·40	13·15	− 0·25

In carrying out the above estimation it is important that the phenyl-
hydrazine solution shall be freshly made up and if it is at all discoloured that
the phenylhydrazine shall be freshly distilled.

Influence of Glucose.

Under the conditions above described the presence of glucose does not
appear to interfere with the estimation of pyruvic acid. In one experiment,

5 cc. of a solution of phenylhydrazine acetate, 10 cc. of a solution of pyruvic acid and 10 cc. of a 1 % glucose solution were made up to 100 cc. and the pyruvic acid estimated as above, and compared with a solution similarly made up but from which the glucose was omitted.

Residual hydrazine (without glucose) required 4·80 cc. N/10 KMnO₄.
    „   „   (with   „)   „   4·85 „   „

The method therefore gives satisfactory results in estimating solutions of concentrations above 0·03 %. Below this concentration probably more accurate results would be obtained by using a more dilute solution of permanganate.

The advantages may be summarised as follows:

(1) It is easily carried out; the whole estimation can be done in little more than an hour.

(2) It gives a greater degree of accuracy than the unsatisfactory method of gravimetric estimation at present in use.

(3) The presence of glucose does not interfere with the estimation.

(4) The method promises to be of general value for the estimation of carbonyl compounds and also for measuring the rate of interaction of these compounds with phenylhydrazine.

REFERENCES.

Fischer, E. (1878), *Annalen*, **190**, 101.
Neuberg and Karczag (1911), *Biochem. Zeitsch.* **36**, 63.
Strache (1891; 1892), *Monatsh.* **12**, 524; **13**, 299.
—— and Kitt (1892), *Monatsh.* **13**, 316.

LX. NOTE ON ISOCHOLESTEROL, COPROSTEROL AND THE CLASSIFICATION OF THE STEROLS.

By CHARLES DORÉE.

(Received Nov. 9th, 1913.)

Isocholesterol was discovered by Schulze [1873] in the fat extracted from raw sheep's wool with ether. The fat is derived from the secretion of the fatty glands surrounding the hair follicle in the skin. For many years the individuality, and even the existence of isocholesterol, were questioned, as many workers, including the present writer, were unable to obtain it. In reply to criticisms of Darmstädter and Lifschütz [1898], Schulze [1898] repeated his original experiments and subsequently Moreschi [1910] obtained the substance from wool fat and fully confirmed the observations of Schulze. In 1910 additional interest was given to the question by the discovery of isocholesterol in a plant product, the so-called South African rubber, which consists of the coagulated latex of various species of Euphorbiaciae. The latex contains about 6 per cent. of rubber and 70 per cent. of resin. The isocholesterol was found as a constituent of the latter (excretory) product [Cohen, 1908]. A careful comparison of the substance with a specimen of the original isocholesterol supplied by Schulze was made by Cohen, and in my opinion no doubt can be entertained as to the identity of the two products and consequently of the recognition of isocholesterol as a definite member of the sterol group.

Coprosterol occurs normally in the faeces of men on ordinary diets, and in those of animals, in place of cholesterol, when diets rich in cholesterol, e.g. raw brain, are given [Dorée and Gardner, 1908]. It is no doubt produced from the cholesterol of the food by changes taking place in the intestine. König and Schluckebier [1908] have stated also that it appears in the excrement of animals which have been kept on diets rich in phytosterol such as peas, maize, coco-nut cake, etc. [Cp., however, Kusumoto, 1908; Dorée and Gardner, 1909.]

The recognition of isocholesterol as a definite member of the sterol group leads me again to point out that while isocholesterol and coprosterol, in their

mode of occurrence and properties, closely resemble one another, they differ in these respects markedly from all the other sterols [Dorée, 1909, 1]. Hitherto the sterols have been classified roughly as zoo- or phyto-sterols according as they were obtained from animal or vegetable sources respectively. But in order to emphasise the special properties and relationships of isocholesterol and coprosterol it is now suggested that a third class should be formed to include them and any other similar substances that may be discovered, and that the terms zoo- and phyto-sterols should be limited to a somewhat more exact definition. Seeing that coprosterol and isocholesterol undoubtedly stand in a close relationship to cholesterol and phytosterol, and that they appear to form a connecting link between these characteristic constituents of animal and vegetable protoplasm, they may be classified as "natural derived sterols" or *metasterols*.

The metasterols occur in the excretions and secretions of animals (intestinal canal, glands of the skin) and of plants (resin). They are never found as constituents of animal or vegetable protoplasm. The connection between them and the other sterols from a biochemical standpoint is shown in the following scheme.
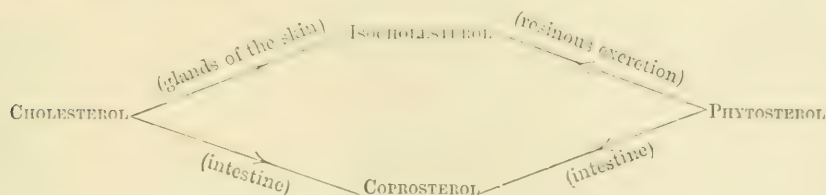
The metasterols are monatomic alcohols of high molecular weight. Unlike the other sterols they (*a*) are saturated towards bromine and hydrogen [Moreschi, 1910], (*b*) do not crystallise from alcohol in plates, (*c*) give the colour tests of Liebermann and Salkowski in a modified way, (*d*) have practically no anti-toxic action towards haemolytic poisons [Hausman, 1905], (*e*) are dextro-rotatory.

It is probable that spongosterol [Henze, 1908], which has no anti-toxic power and apparently is a saturated alcohol, will fall into this class. The position of stigmasterol, $C_{30}H_{48}O$ [Windaus and Hauth, 1906], and brassicasterol, $C_{25}H_{46}O$ [Windaus and Welsch, 1909], found, together with large proportions of ordinary phytosterol in Calabar beans and rape seed respectively, is uncertain.

With the formation of this class it is now proposed to limit the terms zoo- and phyto-sterol to sterols which are found as tissue constituents of

animals and plants respectively. Cohen [1908], in the light of his discovery of isocholesterol as a plant product, has stated that the distinction between zoo- and phyto-sterols can no longer be maintained. But with the proper recognition of isocholesterol as a derivative produced from zoo- or phyto-sterols by metabolic changes, there is now every reason for maintaining it. For in spite of a great number of researches (Hauth [1907] quotes some 50 papers and many more have since appeared), no substance resembling cholesterol has ever been obtained from vegetable protoplasm or one re-sembling phytosterol from animal protoplasm; and this in spite of the fact that herbivorous animals take in phytosterol with their food and the carnivorous plants ingest a cholesterol. If phytosterol taken with the food is used by the animal in building up its tissues, it must first be converted into cholesterol. Furthermore a zoosterol is an invariable constituent of all animal organs and tissues so far examined from Chordata to Coelenterata [Dorée, 1909, 1; Welsch, 1909], and a phytosterol seems similarly to be contained in the tissues of plants of all orders. So fundamental is this distinction considered to be that it has become an important criterion in chemical technology for deciding the animal or vegetable origin of various natural substances. Thus Lewkowitsch [1913] defines these products to be of animal or vegetable origin respectively according to the presence of a zoo- or phyto-sterol in the unsaponifiable portion. [Cf. also the 'phytosterol acetate test.']

The zoo- and phyto-sterols will therefore include such sterols as are found entering into the composition of animal and vegetable protoplasm respectively. Speaking only of those whose properties have been carefully examined (for zoosterols see Dorée [1909, 1] and Welsch [1909]: for phytosterols see Hauth [1907]) the zoo- and phyto-sterols may be held to include "a number of secondary, monatomic alcohols, chiefly $C_{27}H_{46}O$, containing one ethylene linking in the molecule. They crystallise from alcohol in plates. Their benzoates melt at about $145°$ and show the phenomena of liquid crystals. They are all laevo-rotatory and have a powerful anti-haemolytic action towards saponine, etc." Their constant presence in the tissues is in part due to the necessity for the protection of the cells from such toxic substances: their occurrence in the fluids and secretions shows that they are continually being metabolised and, it is believed, conserved by the organism [Dorée and Gardner, 1909].

A summary of the properties of the best known sterols and their derivatives is given in the following table:

(a) Zoosterols:

	M.p.	$[a]_D$	Acetate m.p.	Benzoate m.p.	Dibromide m.p.	Occurrence
Cholesterol	147°	−37°	114°	145°	123°	Universally distributed in the animal kingdom.
Bombicesterol	148°	−35°	129°	146°	111°	In *Bombyx mori* [Menozzi and Moreschi, 1908].
Clionasterol	138°	−37°	133°	143°	114°	In *Cliona celata* [Dorée, 1909, 1].

(b) Phytosterols:

	M.p.	$[a]_D$	Acetate m.p.	Benzoate m.p.	Dibromide m.p.	Occurrence
Phytosterol	137°	−34° [1]	127°	146°	98°	Universally distributed in the Phanerogams.

(c) Metasterols:

	M.p.	$[a]_D$	Acetate m.p.	Benzoate m.p.	Dibromide m.p.	Occurrence
Isocholesterol	137°	+60°	134°	194°	None	Wool fat; resin of Euphorbiaciae.
Coprosterol	100°	+24°	88°	122°	None	Excrement of animals.

[1] In ether solution ; others in chloroform.

Of the chemical relationship existing between cholesterol, phytosterol and the metasterols little is known. On a milk diet, during which bacterial change in the intestine is reduced to a minimum, cholesterol [Müller, 1900] and phytosterol [Windaus, 1908] are not converted to coprosterol. Normally in the case of men, and on diets rich in sterols in the case of animals, coprosterol is produced. Analyses, to which however no great weight can be assigned, seemed to indicate that the coprosterol molecule contained two atoms of hydrogen more than that of cholesterol, and it has been thought that coprosterol was dihydrocholesterol produced by bacterial reduction. Dihydrocholesterol artificially produced [Willstätter and Mayer, 1908] is not however identical with coprosterol, although very similar in its properties as will be seen from the following table :

	M.p.	$[a]_D$	Acetate m.p.	Benzoate m.p.	Ketone m.p.	Observer
Dihydrocholesterol	142°	+29°	111°	155°	128°	Willstätter.
Coprosterol	100°	+24°	88°	122°	63°	Dorée.
Isocholesterol	137°	+60°	134°	194°	?	Cohen.

Observations on a large number of cholesterol and phytosterol derivatives have shown that any modification of the ethylene linking causes a change in the optical rotatory power from negative to positive and at the same time the antihaemolytic function is abolished or reduced to a minimum [Hausman, 1905]. The presumption therefore is strong that it is the side chain containing the double linking in cholesterol that is modified to produce coprosterol. The change however is more than one of simple reduction. The only other explanation that has been offered, based upon the behaviour of a peculiar carbazole derivative of coprostanone [Dorée, 1909, 2], is that the

bacterial action might simultaneously bring about reduction and rearrangement of the unsaturated side chain, producing two methyl groupings, thus:

$$>CH.CH_2.CH:CH_2 + H_2 \qquad >CH.CH<^{CH_3}_{CH_3}$$

Cholesterol   Coprosterol
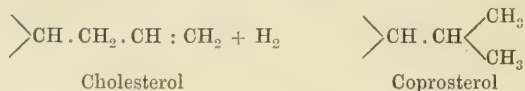
Attempts have been made to solve the problem by studying the oxidation of coprosterol and cholesterol in the hope of obtaining, among the products, a derivative common to both. Coprosterol is difficult to obtain in any quantity, and considering the similarity between coprosterol and isocholesterol and the fact that the raw material from which isocholesterol is obtained is a commercial product, I decided two years ago to work up a large quantity of wool fat in the hope of preparing sufficient isocholesterol to enable a thorough examination of that substance to be made. Quantities of 2 to 3 kilos of wool fat from various sources were saponified under pressure by the method of Lewkowitsch and the mass extracted with ether in the usual way. The unsaponifiable residue was then fractionated in alcohol, acetone, methyl alcohol, etc. None of the fractions appeared to contain isocholesterol and they were therefore severally benzoylated. A number of crystalline benzoates were obtained, but none melting higher than 145°. A quantity of wool fat treated by the method of Darmstädter and Lifschütz also gave a negative result. An explanation of these results may be found in the observation of Cohen [1908] that isocholesterol undergoes a change of the nature of autoxidation on keeping. The somewhat drastic treatments to which commercial wool fat is subjected may therefore have destroyed the isocholesterol. The best method of obtaining it would seem to be to extract fresh clipped raw wool with ether, or to employ the resin of South African rubber.

REFERENCES.

Cohen (1908), *Arch. Pharm.* **246**, 518, 592.
Darmstädter and Lifschütz (1898), *Ber.* **31**, 98, 1126.
Dorée (1909, 1), *Biochem. J.* **4**, 73.
—— (1909, 2), *J. Chem. Soc.* **95**, 655.
—— and Gardner (1908), *Proc. Roy. Soc.* B. **80**, 230.
—— —— (1909), *Proc. Roy. Soc.* B, **81**, 109.
Hausman (1905), *Beiträge*, **6**, 567.
Hauth (1907), *Zur Kenntnis der Phytosterine*, Diss. Freiburg.
Henze (1908), *Zeitsch. physiol. Chem.* **55**, 427.

König and Schluckebier (1908), *Zeitsch. Nahr. Genussm.* **15**, 651.

Kusumoto (1908), *Biochem. Zeitsch.* **142**, 407.

Lewkowitsch (1913), *The Chemical Technology and Analysis of the Oils, Fats and Waxes*, 3rd Ed. Macmillan and Co.

Menozzi and Moreschi (1908), *Atti R. Accad. Lincei* [v], **17**, ii, 95.

Moreschi (1910), *Atti R. Accad. Lincei* [v], **19**, ii, 53–57.

Müller (1900), *Zeitsch. physiol. Chem.* **29**, 129.

Schulze (1873), *J. pr. Chem.* **7**, 169.

—— (1898), *Ber.* **31**, 1200.

Welsch (1909), *Vorkommen der Sterine in Tier. u. Pflanzenreich*, Diss. Freiburg.

Willstätter and Mayer (1908), *Ber.* **41**, 2199.

Windaus (1908), *Arch. Pharm.* **246**, 121.

—— (1911), *Die Sterine, Biochemisches Handlexikon* (Abderhalden), **3**, 268.

—— and Hauth (1906), *Ber.* **39**, 4378.

—— and Welsch (1909), *Ber.* **42**, 612.

LXI. THE HYDROLYSIS OF GLYCOGEN BY DIASTATIC ENZYMES. II. THE INFLUENCE OF SALTS ON THE RATE OF HYDROLYSIS. (Preliminary Communication.)

By ROLAND VICTOR NORRIS, *Beit Memorial Research Fellow.*

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In a recent communication [Norris, 1912] it was pointed out that samples of glycogen prepared from different animals were hydrolysed at different rates by pancreatic amylase. In view, however, of the fact that these preparations contained varying amounts of salts it seemed desirable to examine how far this might influence the rate of hydrolysis, and a quantitative study has therefore been made of the action of neutral salts on diastatic action. The results quickly showed that the experiments referred to were in no way invalidated by the varying salt content, the latter in every case being sufficient to produce the maximum degree of hydrolysis and not great enough to cause any inhibition.

The experiments have however been continued in the hope of finding some explanation of the manner in which salts exert their influence.

It has been known for some time that a dialysed amylase solution when added to a starch solution free from salts produces but little hydrolysis, and that on adding certain salts in small quantities the activity of the enzyme is restored. In spite of a considerable amount of work on this point, the explanation of this fact is not by any means clear. It is agreed that the most active salts are those of the halogen acids. With regard to sulphates, however, very divergent results have been obtained. Cole [1906, 1], for example, states that sulphates accelerate the action while Grützner [1902] maintains that magnesium and sodium sulphate are "specific poisons" for the ferment. In this connection however it must be pointed out that in one at least of Cole's experiments the addition of sulphate produced no acceleration although the concentration of the salt was similar to that used in previous experiments in which an acceleration had been obtained. These and other divergencies

may perhaps be partly explained by one of the following reasons. Firstly, the source and method of preparation of the enzyme has differed with nearly every worker. Hence while some investigators have employed solutions containing only traces of proteins, in other cases these have been present in considerable quantity. Again, in the few cases where the action has been followed quantitatively, the diastatic activity has been estimated either by Roberts' achromic point method [Roberts, 1891] or by the method of Wohlgemuth [1908, 1], and it has been shown by Evans [1912, 1] that neither of these is satisfactory. Finally, in some cases, the enzyme and starch solution employed have been by no means free from salts, the controls all showing a marked degree of hydrolysis, that is to say the observed effect was really due to a mixture of salts and not alone to the particular salt under investigation.

In the following experiments glycogen has been employed instead of starch, while the enzyme has consisted of an extract of pigs' pancreas.

Preparation of glycogen. This was obtained from dogs' liver by Pflüger's method. The crude glycogen was purified by repeated precipitation of its solution by alcohol and was finally dialysed for a week, the last three days' dialysis being against running distilled water.

Preparation of enzyme. This consisted of a Buchner extract of pigs' pancreas which was dialysed for three days, in the course of which a certain amount of protein usually separated out. The dialysed extract was then filtered till perfectly clear and diluted from ten to twenty times with distilled water.

Experimental methods. The following may be described as typical of the method employed.

A 1 per cent. or 2 per cent. solution of glycogen was as a rule used and to this was added a suitable concentration of the salt under investigation. The mixture was then brought to a temperature of 37° in a thermostat and the enzyme added. After 15 minutes and 30 minutes hydrolysis, 20 cc. of the mixture were removed and the sugar immediately estimated by Bertrand's method. The sugar solution was added directly to the alkaline copper mixture and hence the hydrolytic action stopped instantaneously. The strength of enzyme used was adjusted so that the readings taken fell on the linear portion of the hydrolysis curve. [See Evans, 1912, 2 and Norris, 1912.] The salts employed were nearly all Kahlbaum's "for analysis with certificate of guarantee."

Effect of dialysis on glycogen hydrolysis.

As in the case of starch the result of dialysis of both enzyme and glycogen resulted in almost complete inactivation. Similarly the hydrolytic power was again restored by the addition of certain salts.

Influence of salts.

Under this heading neutral salts only are considered. The rate of hydrolysis is of course greatly influenced by any change in the reaction of the medium, but this point has been dealt with in a previous communication [Norris, 1912].

Hydrogen ion determinations made on glycogen solutions containing varying concentrations of sodium chloride showed that the reaction of the medium was not altered by the presence of this salt in the concentrations employed, hence the accelerating effect of sodium chloride is not due to this cause.

Sodium chloride.

A series of mixtures was made up each containing 1 per cent. glycogen and a concentration of sodium chloride ranging from zero to 0·003 N. These were in turn incubated with 1 cc. of a dilute enzyme preparation and hydrolysis allowed to proceed for 15 minutes. The sugar in 20 cc. of each solution was then estimated.

The results are shown in Fig. 1 where the abscissae represent the cc. of 0·1 N NaCl in 100 cc. of the mixture and the ordinates the cc. of $KMnO_4$ used in the sugar estimation.

cc. N/10 NaCl in 100 cc. reaction mixture.
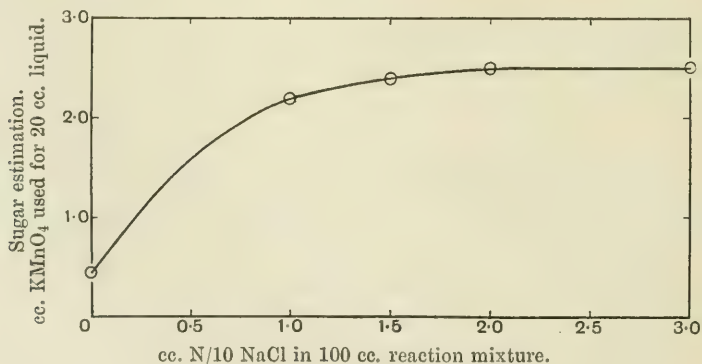
Fig. 1.

The results show that there is a rapid increase in the hydrolysis with increasing NaCl content until the latter reaches a concentration of about 0·002 N (0·012 per cent.). This value is in close agreement with Cole's results with starch and ptyalin.

On further addition of salt there is at first no change in rate, but with high concentration a slight retardation may be produced.

An investigation was next undertaken to decide whether the value of this optimum concentration (0·002 N) would be changed by alteration in the concentration of either glycogen or enzyme. On this point results have been somewhat contradictory but it seems probable that the glycogen concentration has but little influence on the amount of salt required to produce the maximum rate of hydrolysis. The optimum salt content for 2 per cent. glycogen has usually been about 0·002 N, that is to say the same as for 1 per cent. glycogen.

On increasing the amount of enzyme, however, a higher concentration of NaCl is usually required. This is in agreement with the results of Cole [1906. 1] and Starkenstein [1910]. The latter, working with starch. states that the amount of NaCl necessary to give the maximum rate of hydrolysis varies *directly* with the concentration of enzyme and has even based on this a method for the estimation of diastase in animal organs [Starkenstein, 1912]. The results of my experiments do not point to such a simple relationship, though as already stated more NaCl is usually required with increased concentration of enzyme. It must be remembered that it is usually in the enzyme that impurities such as proteins etc. will be found and the latter may begin to play an important part when the amount of enzyme present is not small.

The following table shows the results of one experiment in which the concentration of enzyme was twice that usually employed.

TABLE I.

Determination of NaCl optimum with 1 per cent. glycogen and high enzyme concentration.

Experiment	Concentration of NaCl	Percentage hydrolysis	
		15 mins.	30 mins.
A	0	5·5	9·20
B	0·002 N	15·1	26·5
C	0·004 N	16·5	28·0
D	0·006 N	18·3	29·8
E	0·01 N	18·3	29·5

In this case therefore by doubling the concentration of enzyme the optimum salt concentration was raised from 0·002 N to 0·006 N, that is to say three times.

In other experiments however very much lower results were obtained and there seems to be some unknown factor concerned. The point is still under investigation.

Comparison of sodium chloride with other salts.

Chlorides of different metals.

Table II shows the accelerating effect of the chlorides of sodium, potassium, calcium, barium and magnesium.

These were added in insufficient quantity to produce the optimum rate of hydrolysis, so that any variation in their accelerating power could be detected.

TABLE II.

Accelerating power of various chlorides. 1 per cent. glycogen.
Concentration of salt = 0·0005 N.

		Percentage hydrolysis	
Salt		15 mins.	30 mins.
	NaCl	10·83	18·76
	KCl	10·61	18·8
	CaCl$_2$	10·70	—
	BaCl$_2$	10·61	17·6
	MgCl$_2$	10·61	18·52
Control	0	0·68	1·14

All the above salts are therefore of equal accelerating power, that is to say the kation exerts practically no influence on the reaction. This is in agreement with the results of Starkenstein [1910]. Cole [1906, 1] considered that the anion accelerated while the kation depressed the action. If the latter were correct, however, one would expect the chlorides containing a divalent kation to be less active than sodium or potassium chloride and as shown this is not the case. The view that the anion is the more important factor is further strengthened by a comparison of the accelerating power of chlorides, bromides and iodides. The results given in Table III confirm those of previous investigators, namely, that the acceleration decreases in the order given, the drop from bromide to iodide being much greater than that from chloride to bromide.

TABLE III.

Comparison of KCl, KBr and KI. (Concentration of salt = 0·0005 N.)

	Percentage hydrolysis	
Salt	15 mins.	30 mins.
KCl	10·61	18·80
KBr	8·31	15·60
KI	2·5	3·70
Control	0·68	1·40

Influence of sulphates.

The influence of three sulphates has been investigated but it has been found that they have no accelerating power at all; these results are therefore in agreement with those of Wohlgemuth [1908, 2] but in opposition to those of Cole [1906, 1]. The latter however found that sulphates were much less effective than the halogen compounds. These results are of some interest, as one would expect if the anion were the factor concerned, that the divalent anion would be extremely potent. The situation is complicated, however, as pointed out by Cole, by the fact that sodium sulphate in moderate dilutions chiefly dissociates into Na^+ and $NaSO_4^-$ in which case the anion is monovalent.

On the other hand it has not been found that sulphates have any depressing action [cf. Grützner, 1902], nor do they hinder the acceleration produced by NaCl etc.

TABLE IV.

Influence of sulphates. 1 per cent. glycogen.

	Salt	Concentration of salt	Percentage hydrolysis	
			15 mins.	30 mins.
1.	0	Control	1·60	5·04
	Na_2SO_4	0·002 N	1·65	5·0
	Na_2SO_4	0·01 N	1·55	5·0
	$MgSO_4$	0·001 N	1·60	—
2.	$MgSO_4$ + NaCl	Each 0·001 N	9·10	16·01
	NaCl	0·001 N	9·20	16·10

For the sake of comparison the results obtained with different salts have been collected in Table V.

TABLE V.

Comparison of various salts.   1 per cent. glycogen containing 0·0005 N salt.

Salt	Percentage hydrolysis		Remarks
	15 mins.	30 mins.	
0 (Control)	0·68	1·14	
NaCl	10·83	18·76	Halogen salts.
KCl	10·61	18·8	Both ions monovalent.
KBr	8·31	15·6	
KI	2·5	3·7	
CaCl$_2$	10·70	—	Halogen salts with divalent kation.
BaCl$_2$	10·61	17·6	
MgCl$_2$	10·61	18·5	
KNO$_3$	2·75	5·08	Both ions monovalent.
La(NO$_3$)$_3$	2·75	4·94	Trivalent kation.
Na$_2$SO$_4$	0·55	1·0	Divalent anion.
K$_2$SO$_4$	0·64	1·20	,, ,,
MgSO$_4$	0·70	—	Both ions divalent.

It will be seen from the results tabulated above that the only salts of those tried which have a powerful accelerating action are those of the halogen acids, although nitrates have a small influence. It is also clear that the anion is much more concerned in the reaction than is the kation. Further it is probable that the action of the salts is chiefly confined to the enzyme, for it has been shown by Cole [1906, 2] that in the action of invertase, where the substrate is not a colloidal solution, salts have again a powerful influence. In this case, however, the action is reversed, that is to say, the hydrolysis is retarded by chlorides.

If the function of the anion be simply to alter the charge on the enzyme, it is difficult to understand why sulphates have no accelerating effect, for sulphates discharge a ferric hydroxide solution much more readily than chlorides. The fact that the number of salts producing an acceleration is so restricted, however, points to some other explanation.

In the meantime it seems desirable to examine separately the effect of salts on the glycogen and enzyme respectively from the point of view of adsorption and charge, and experiments on these lines are in progress but are not as yet sufficiently advanced to furnish an explanation. The action of a further series of salts is also being investigated.

SUMMARY.

(1) A dialysed glycogenase (pancreatic) solution has practically no hydrolysing action when added to a dialysed glycogen solution.

(2) The activity of the enzyme is restored by the addition of small quantities of certain salts.

(3)   The most powerful of these are the salts of the halogen acids, the activity diminishing in the order chlorides, bromides, iodides; nitrates have also a slight accelerating action.

(4)   Sulphates do not restore the activity of a dialysed enzyme solution, neither are they inhibitors [cf. Grützner, 1902].

(5)   The concentration of salt required to produce a maximum degree of hydrolysis rises with increasing enzyme concentration but appears to be independent of the glycogen concentration within the limits tried.

(6)   The anion is probably the part of the salt concerned in the acceleration, the nature of the kation (valency) having no influence.

REFERENCES.

Cole (1906, 1), *J. Physiol.* **30**, 202.
—— (1906, 2), *J. Physiol.* **30**, 281.
Evans (1912, 1), *J. Physiol.* **44**, 220.
—— (1912, 2), *J. Physiol.* **44**, 191.
Grützner (1902), *Pflüger's Archiv*, **91**, 195.
Norris (1912), *Biochem. J.* **7**, 26.
Roberts (1891), *Digestion and Diet*, London, p. 68.
Starkenstein (1910), *Biochem. Zeitsch.* **24**, 210.
—— (1912), *Biochem. Zeitsch.* **47**, 300.
Wohlgemuth (1908, 1), *Biochem. Zeitsch.* **9**, 1.
—— (1908, 2), *Biochem. Zeitsch.* **9**, 10.

LXII. THE ENZYMATIC FORMATION OF POLY-SACCHARIDES BY YEAST PREPARATIONS.

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Two reasons led to the institution of the following experiments. In an earlier paper [1904] the authors showed that the carbon dioxide evolved in the alcoholic fermentation of sugars by yeast juice was not equivalent to the sugar which disappeared from the solution, and ascribed this fact to the production of a hydrolysable compound of low reducing power. It was subsequently found that in alcoholic fermentation by yeast preparations a certain amount of hexosephosphate is formed, which has a lower reducing power than the sugar (about 75 per cent.) from which it is formed and would therefore in part account for the phenomenon.

In the second place it appears to follow from the authors' equations of fermentation [1908] that in the normal fermentation both of fructose and glucose half the sugar passes through the form of hexosephosphate, which is then hydrolysed. Since these two hexoses appear to yield the same hexose-phosphate it would be expected that as the fermentation proceeded fructose and glucose alike would be partially converted into the same product of hydrolysis, and the rotations of their solutions should therefore tend to approximate to each other. The exact nature of the substance produced along with phosphoric acid by the hydrolysis of hexosephosphate in yeast juice is not definitely known, but all the evidence points towards its being fructose. Hence we should expect in all fermentations of glucose by yeast or yeast preparations a progressive conversion of glucose into fructose [compare Slator, 1911]. This however has not been observed and experiments were therefore made on the subject, especially with the object of ascertaining whether the product of hydrolysis of the hexosephosphate underwent any secondary change, such as condensation to a polysaccharide.

The present experiments show that both from glucose and fructose one or more dextrorotatory polysaccharides are produced during alcoholic

fermentation by yeast preparations. The previous conclusion of the authors is therefore confirmed, but it is not yet settled whether the polysaccharide formation takes place at the expense of the glucose and fructose themselves or occurs indirectly as the result of the action of some enzyme on the product of hydrolysis of the hexosephosphate. Further investigations on this point are in progress.

It is well known that living yeast forms glycogen when brought into excess of sugar solution [see Pavy and Bywaters, 1907], and the behaviour of yeast preparations therefore indicates that the enzymes involved in this synthesis are probably, at least to some extent, still present and active. The isolation of a substance having the qualitative reactions of glycogen is a further confirmation of the observation of Cremer [1899] who found that in yeast juice free from glycogen a substance was slowly formed in the presence of sugar which gave the characteristic glycogen reactions.

Experimental.

Experiment 1. Three lots of 100 cc. maceration juice (from Schroder's dried Münchener yeast) were incubated at 25° with toluene until they had attained the temperature of the bath. To Nos. 1 and 2 were then added 25 cc. of a 40 per cent. solution of glucose and the evolution of carbon dioxide observed. At the same time No. 3 was boiled and cooled, and 25 cc. of the same glucose solution added.

In Nos. 1 and 2 a maximum rate of 21·6 cc. per 2 minutes was slowly attained, which then rapidly diminished until in 52 minutes a constant rate of 2·8 cc. per 2 minutes was reached. The initial high rate was due to the presence of free phosphate in the maceration juice, which was converted into hexosephosphate. During this period the total gas evolved was 293·6 cc. at room temperature and pressure. After 52 minutes No. 2 was boiled, whilst the fermentation in No. 1 was allowed to proceed for 17 hours 38 minutes, during which time 1458 cc. of CO_2 had been evolved. No. 1 was then boiled. The contents of all three flasks were then filtered, and the free phosphate estimated in an aliquot portion of each. The amount of glucose in each was determined by precipitating the proteins in aliquot portions with Patein's mercuric nitrate solution and estimating the glucose by means of Pavy's method.

The treatment with mercuric nitrate precipitates the hexosephosphate, so that in order to determine the amount of sugar used up allowance must be made for the quantity bound up in the form of hexosephosphate. This is

readily done since it has given rise to the CO_2 equivalent to the free phosphate present at the beginning, and can therefore be determined by subtracting the carbonic acid corresponding with the constant rate of fermentation from the total actually evolved, in the manner frequently described before.

Free phosphate in No. 3 = 1·110 g., in No. 2 = 0·130 g. and in No. 1 = 0·127 g. $Mg_2P_2O_7$, showing that the same quantity of sugar is still bound up as hexosephosphate in No. 1 as in No. 2.

CO_2 evolved up to the time when No. 2 was boiled (after 52 mins. fermentation)	...	...	...	293·6 cc.
Rate = 2·8 × 26 ...	...	...	...	72·8 „
Equivalent of phosphate	...	...	...	220·8 cc. or 201 cc. at N.T.P., i.e. 0·394 g.

This corresponds therefore to 0·79 g. glucose.

The amount of glucose converted into hexosephosphate may also be determined from the phosphate combined during the experiment. Phosphate bound up in No. 2 = 1·110 − 0·130 = 0·980 g. $Mg_2P_2O_7$; equivalent therefore to $0·980 \times \frac{180}{222} = 0·795$ g. glucose.

The tables show the amount of glucose which cannot be accounted for as CO_2 and alcohol or as hexosephosphate, the glucose originally present being obtained from No. 3 by analysis.

Flask (2).

CO_2 evolved = 293·6 cc. or 267 cc. at N.T.P. = 0·53 g. equivalent to 1·06 g. glucose.

Glucose bound up as hexosephosphate	...	...	0·79 g.	„
Total accounted for	...	...	...	...	1·85 g.	„

Original glucose	9·98 g.
Final glucose	8·15 g.
Actual loss	1·83 g.

No disappearance of glucose was observed.

Flask (1).

CO_2 evolved = 1458 cc. or 1339 cc. at N.T.P. or 2·634 g. equivalent to 5·27 g. glucose.

Glucose bound up as hexosephosphate	...	...	0·79 g.	„
Total accounted for	...	...	...	...	6·06 g.	„

Original glucose	9·98 g.
Final glucose	0·91 g.
Actual loss	9·07 g.

Thus 9·07 − 6·06 = 3·01 g. of glucose have disappeared.

The ratio between the reducing power and the optical rotation was determined in each mixture after the treatment with Patein's solution in

order to see if any active substance other than sugar were present. For the sake of convenience the rotation observed in a 400 mm. tube is compared with the reducing power determined by Pavy's method expressed as grams of glucose in 100 cc.

With pure glucose this ratio $\frac{\text{Rotation in 400 mm. tube}}{\text{Reduction (g. glucose per 100 cc.)}} = + 2.05$, whilst with pure fructose it is -4.03.

These ratios were found to be

$$(1) \quad + \frac{2.149}{0.120} = + 17.91.$$

$$(2) \quad + \frac{2.224}{1.08} = + 2.06.$$

$$(3) \quad + \frac{2.843}{1.32} = + 2.15.$$

It is thus seen that in No. 1 some substance is present which has a much greater dextrorotatory power than has glucose, whereas in No. 2 all the rotatory power may be accounted for by the quantity of glucose present.

Experiment 2. A similar experiment was carried out with fructose (Kahlbaum), the following mixtures being employed:

(1) 100 cc. maceration juice + 25 cc. 40 per cent. fructose + toluene.
(2) 100 cc.   „   „   + 25 cc. 40 per cent.   „   „
(3) 100 cc.   „   „   + 25 cc. water + toluene.
(4) 100 cc.   „   „   + 25 cc.   „   „

The juice in each case was kept in the bath until the temperature was attained, and the fructose and water then added. Nos. 1 and 3 were boiled immediately, whilst the others were incubated and the fermentation observed. In No. 2, a high phosphate rate of 53 cc. per 5 minutes was rapidly reached which then decreased as usual to a constant rate of 7·5 cc. per 5 minutes; at the end of 17 hours both Nos. 2 and 4 were boiled. During the first 70 minutes No. 2 had given off 352·5 cc. of carbon dioxide, the amount due to the phosphate thus being $352.5 - 14 \times 7.5 = 352.5 - 105 = 247.5$ cc.; the total evolved in the 17 hours was 1179·5 cc. at 762·8 mm. and 15°. No. 4 showed no fermentation at all.

A portion of each of the four filtered mixtures was treated with Patein's solution as in the last experiment, and the reducing power and rotations determined in an aliquot portion of the filtrate. The figures given are calculated for the total volume of the juice.

	1. Original mixture	2. After 17 hrs.	3. Original juice alone	4. Juice incubated alone
Rotation in 400 mm. tube	$-30.81°$	$+4.65°$	$-0.061°$	$-0.066°$
Total sugar as g. glucose	9·31	1·10	0	0

Loss of sugar $= (1) - (2) = 8\cdot21$ g.

CO_2 evolved $= 1179\cdot5$ cc. at $762\cdot8$ mm. and $15° = 2\cdot08$ g.

CO_2 corresponding to fructose bound up as hexosephosphate $= 247\cdot5$ cc. at $762\cdot8$ mm. and $15° = 0\cdot41$ g.

Total sugar accounted for as CO_2 and hexosephosphate therefore

$$= 2 \times 2\cdot49 = 4\cdot98 \text{ g.}$$

Sugar disappeared $= 8\cdot21 - 4\cdot98 = 3\cdot23$ g.

The ratios of rotation to reduction expressed as before were also determined in the solution after treatment with Patein's solution and were found to be (1) $-4\cdot14$, (2) $+5\cdot28$; pure fructose $= -4\cdot03$.

It is thus seen that in this experiment a substance having a high dextrorotation was formed, so that although all the fructose was not used up, the mixture had changed in rotation from laevorotatory to dextrorotatory, whilst a much larger proportion of the original fructose had disappeared than could be attributed to the fermentation.

In the solutions before treatment with Patein's solution the free phosphate was estimated and was found to be:

(1) $1\cdot203$ g. $Mg_2P_2O_7$.
(2) $0\cdot174$ g.   „
(3) $1\cdot210$ g.   „
(4) $1\cdot208$ g.   „

From this it is seen that the phosphate and hence an equivalent portion of the sugar was still bound up as hexosephosphate at the end of the experiment (No. 2). These numbers serve as before as a check on the quantity of sugar which has been converted to hexosephosphate, viz. that amount corresponding to

$1\cdot203 - 0\cdot174 = 1\cdot029$ g. $Mg_2P_2O_7$ or $1\cdot029 \times \frac{180}{222}$ fructose $= 0\cdot834$.

Fructose calculated from equivalent of CO_2 as above $= 0\cdot41 \times 2 = 0\cdot82$ g.

The mixtures before treatment with Patein's solution were tested with iodine solution; Nos. 1, 3, and 4 gave no colouration, whereas No. 2 gave a deep reddish brown colouration.

A portion of No. 2 treated with three volumes of alcohol gave a white precipitate, which was redissolved in water and again precipitated with alcohol. This last precipitate gave an opalescent solution in water which was precipitated by saturation with ammonium sulphate and gave a red colouration with iodine. The only difference which could be seen from the behaviour of glycogen was that it gave a somewhat different red colour with iodine.

The other solutions Nos. 1, 3, and 4 gave slight precipitates with alcohol, the aqueous solutions of which gave however no colouration with iodine.

It is thus seen that during the fermentation of fructose by maceration juice a dextrorotatory, glycogen-like substance is formed.

These results appear to us as already indicated to throw some light on the cause of the difference which exists between sugar fermented and carbon dioxide evolved, not only in the case of yeast preparations but also in that of living yeast. Euler and his colleagues in recent papers have argued from the existence of this difference between the amount of sugar actually removed by living yeast from a glucose solution and the amount equivalent to the CO_2 evolved, which he terms $\Delta - C$, that the hexose requires to undergo some change which renders it directly fermentable and that the difference $\Delta - C$ represents the amount which is in this intermediate condition. [Euler and Johannson, 1912; Euler and Berggren, 1912.] There seems however to be no good reason to suppose that Euler and Johannson's $\Delta - C$ cannot be accounted for by the well-known formation of glycogen which has been shown by Pavy and Bywaters [1907] to be of the order of magnitude required.

In Euler and Berggren's experiments on the effect of yeast extract in increasing both rate of fermentation and $\Delta - C$ [1912], no counts of yeast cells before and after the experiments were made. As the earliest observations were made after an hour at $15°-18°$ and the experiments in some cases extended to over six hours (1 g. pressed yeast in 25 cc. of solution), the possibility of yeast growth must not be overlooked. This is still more probable in the cases in which only 0·25 g. of pressed yeast was taken and tested with yeast extract itself, various precipitates from yeast extract, and with sodium nucleinate or ammonium formate [1912, pp. 216, 217: Euler and Cassel, 1913], in a total volume of 40 cc.

An experiment made on this point showed that under similar conditions of concentration growth readily occurs at $25°$. The yeast was added as 5 cc. of a suspension of 5 g. yeast in 100 cc. H_2O, i.e. 0·25 g. yeast.

(1) and (2)   5 cc. yeast suspension + 20 cc. of 20 per cent. glucose + 15 cc. H_2O.

(3) and (4)   5 cc. yeast suspension + 20 cc. of 20 per cent. glucose + 15 cc. yeast extract.

In 345 mins. the evolutions were respectively 61, 59·6, 146·3, 150 cc. At the close of this time the numbers of cells present per cc. were $68·7 \times 10^6$, $68·5 \times 10^6$, $105·25 \times 10^6$, $98·8 \times 10^6$.

Asparagine acts in a precisely similar manner, 0·25 g. added to 0·5 g. yeast in 30 cc. sugar solution increasing the evolution in 2 hrs. at 25 from 73·6 to 89·4 cc.

. It therefore seems that the experiments in which Euler has shown the accelerating effect of yeast extract, sodium nucleinate, etc., on the action of living yeast require revision from this point of view.

The method of testing for a co-enzyme by the action of solutions on living yeast is moreover open to the criticism that the yeast cell is, if at all, only imperfectly permeable to the co-enzyme so that negative results would be of little value.

SUMMARY.

During the alcoholic fermentation of glucose and fructose by Lebedeff's maceration extract of dried yeast, dextrorotatory polysaccharides are produced, and it is to the formation of these that the difference between the sugar removed and that equivalent to the carbon dioxide evolved is principally to be attributed.

REFERENCES.

Cremer, M. (1899), *Ber.* **32**, 2062.
Euler and Berggren (1912), *Zeitsch. Gärungsphysiol.* **1**, 203.
—— and Cassel (1913), *Zeitsch. physiol. Chem.* **86**, 122.
—— and Johannson (1912), *Zeitsch. physiol. Chem.* **76**, 347.
Harden and Young (1904), *Ber.* **37**, 1052.
—— —— (1908), *Proc. Roy. Soc.* B, **80**, 299.
Pavy and Bywaters (1907), *J. Physiol.* **36**, 149.
Slator, A. (1911), *J. Inst. Brewing*, **17**, 147.

NOTE:

HASLAM. *Separation of proteins*, Part III, Globulins.   This Journal 1913, **7**, 492.

In section 4 of Summary, p. 515, *for* 0·1 mg. P $^0/_0$ *read* 0·1 P $^0/_0$.

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